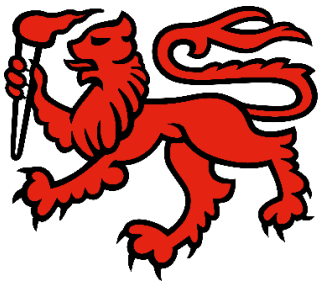

UNIVERSITY *of* TASMANIA

The Role of Zinc and the Zip7 Transporter in Disease Processes Associated with IR in Skeletal Muscle

Shaghayegh Norouzi

MSc (Biochemistry) Kharazmi University of Tehran

BSc (Biology) Chamran University of Ahvaz

A thesis submitted in fulfilment of requirements for the
Degree of Doctor of Philosophy

School of Health Sciences

University of Tasmania

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Publications and Statement of Co-Authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Candidate	Shaghayegh Norouzi, College of Health and Medicine, School of Health Sciences, University of Tasmania
Author 1 (Supervisor)	Dr. Stephen Myers, College of Health and Medicine, School of Health Sciences, University of Tasmania
Author 2 (Co-supervisor)	Dr. Sukhwinder Singh Sohal, College of Health and Medicine, School of Health Sciences, University of Tasmania
Author 3	John Adulcikas, College of Health and Medicine, School of Health Sciences, University of Tasmania
Author 4	Dr. Darren Henstridge, College of Health and Medicine, School of Health Sciences, University of Tasmania, and Baker Heart and Diabetes Institute, Victoria.
Author 5	Dr. Sabrina Sonda, College of Health and Medicine, School of Health Sciences, University of Tasmania

Author details and their roles:

Paper 1: Zinc transporters and insulin resistance: therapeutic implications for type 2 diabetes and metabolic disease

Shaghayegh Norouzi, John Adulcikas, Sukhwinder Singh Sohal and Stephen Myers

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Location in thesis: Chapter 2

Candidate was the primary author, and along with author 1, contributed to the conception and design of the research project, and drafted parts of the article. Candidate contributed approximately 80% to the planning, execution and preparation of the work for the article. Author 2 and 3 contributed to the review of the manuscript.

Paper 2: Zinc stimulates glucose oxidation and glycemic control by modulating the insulin signaling pathway in human and mouse skeletal muscle cell lines

Shaghayegh Norouzi, John Adulcikas, Sukhwinder Singh Sohal, Stephen Myers

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Candidate was the primary author and contributed approximately 80% to the planning, execution and preparation of the work for the article and conducted all the experiments. Candidate along with author 1 contributed to the conception and design of the research project, and drafted parts of the article. Author 2 and 3 contributed to the review of the manuscript.

Paper 3: The zinc transporter Zip7 is downregulated in the skeletal muscle of insulin resistant cells and high fat fed mice

Shaghayegh Norouzi, John Adulcikas, Darren Henstridge, Sabrina Sonda, Sukhwinder Singh Sohal and Stephen Myers

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Location in thesis: Chapter 5

Candidate was the primary author and contributed approximately 80% to the planning, execution and preparation of the work for the article and conducted all the experiments. Candidate along with author 1 contributed to the conception and design of the research project, and drafted parts of the article. Author 2 supplied the High Fat Diet mice tissues, and data for weight measurements, fat mass and glucose tolerance. Authors 2, 3, 4 and 5 contributed to the overall review of the manuscript.

We, the undersigned, endorse the above stated contribution of work undertaken for each of the published peer-reviewed manuscripts contributing to this thesis:

Signed:

Shaghayegh Norouzi	Dr. Stephen Myers	Professor Nuala Byrne
Candidate	Primary supervisor	Head of the School
School of Health Sciences	School of Health Sciences	School of Health Sciences
University of Tasmania	University of Tasmania	University of Tasmania
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1. Adulcikas, John, Sabrina Sonda, **Shaghayegh Norouzi**, Sukhwinder Singh Sohal, and Stephen Myers. "Targeting the Zinc Transporter ZIP7 in the Treatment of Insulin Resistance and Type 2 Diabetes." *Nutrients* 11, no. 2 (2019): 408.
2. Adulcikas, John, **Shaghayegh Norouzi**, Lawrence Bretag, Sukhwinder Singh Sohal, and Stephen Myers. "The zinc transporter SLC39A7 (ZIP7) harbours a highly-conserved histidine-rich N-terminal region that potentially contributes to zinc homeostasis in the endoplasmic reticulum." *Computers in biology and medicine* 100 (2018): 196-202.
3. Gundamaraju, Rohit, Ravichandra Vemuri, Wai Chong, Stephen Myers, **Shaghayegh Norouzi**, Madhur Shastri, and Rajaraman Eri. "Interplay between Endoplasmic Reticular Stress and Survivin in Colonic Epithelial Cells." *Cells* 7, no. 10 (2018): 171.
4. Stephen Myers, Madhur D Shastri, John Adulcikas, Sukhwinder Singh Sohal, and **Shaghayegh Norouzi**. "Zinc and gastrointestinal disorders: A role for the zinc transporters Zips and ZnTs." *Current pharmaceutical design* 23, no. 16 (2017): 2328-2332.
5. Is there a relationship between zinc and type 2 diabetes? Stephen Myers and **Shaghayegh Norouzi**. 2016, *Journal of clinical diabetes and practice* 1:3.

Presentations at Conferences during PhD Candidature

Zinc induces insulin signalling cascade in human skeletal muscle cell line.
Shaghayegh Norouzi, John Adulcikas, Sukhwinder Sohal and Stephen Myers.
Insulin Resistance, Diabetes & Cardiovascular Disease, December 2017.
Los Angeles, America

The effects of zinc on insulin resistance.

Shaghayegh Norouzi
Rural health and collaborative research symposium, June 2017.
Launceston, Australia

Zinc regulates molecules implicated in insulin signaling: implications for insulin resistance.

Shaghayegh Norouzi and Stephen Myers.

Medical, Medicine and Health Sciences, December 2016.

Melbourne, Australia

Zinc is critical for the activation of cell signalling molecules in skeletal muscle: implications for insulin resistance and type 2 diabetes.

Shaghayegh Norouzi and Stephen Myers.

Tasmanian Health Research Conference, July 2016.

Launceston, Australia

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LIST OF ABBREVIATIONS

AKT	Protein kinase B
AVV	Adeno-associated viral vector
ALPs	Alkaline phosphatases
CREB	cAMP response element binding protein
CK2	Casein kinase II
cDNA	Complementary DNA
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EZS	Early zinc signalling
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated protein kinase
FG	Fasting glucose
FCS	Fetal calf serum
Gbe	Glycogen branching enzyme
GFP	Green fluorescent protein
GLUT4	Glucose transporter 4
GOx	Glucose oxidase
GRB2	Growth factor receptor-bound protein 2
GS	Glycogen synthase
GSK-3β	Glycogen synthesis kinase-3β
GPCR	G-protein coupled receptor
GWAS	Genome-wide association studies
HOMA-B	Pancreatic beta-cell function
Hras	Harvey rat sarcoma virus oncogene
HFD	High fat diet

IGF-1R	Insulin-like growth factor-1 receptor
Ir	Insulin receptor
Irs1	Insulin receptor substrates 1
Irs2	Insulin receptor substrates 2
IR	Insulin resistance
IRSs	Insulin receptor substrates
IDF	International Diabetes Federation
IVE	*In vivo* electroporation
Kras	Kirsten rat sarcoma viral oncogene homolog
LB	Lysogeny broth
LZS	Late zinc signalling
MAGIC	Meta-Analysis of Glucose and Insulin-related traits Consortium
MAPK	Mitogen-activated protein kinase
Mek	Mitogen-activated protein kinase kinase
MT	Metallothionein
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
NTB1	Non-transferrin bound iron
NaPy	Sodium pyrithione
PI3K	Phosphoinositide 3 kinase
PCOS	Polycystic ovary syndrome
PDK1	Pyruvate Dehydrogenase Kinase 1
PEPCK	Phosphoenolpyruvate carboxykinase
PIS	Protease Inhibitor Solution
PKB	Protein kinase B
Pparg	Peroxisome proliferator activated receptor gamma
PRAS40	Proline-rich AKT substrate of 40 kDa
PTHrP	Parathyroid hormone-related peptide
PTP1B	Protein tyrosine phosphatase 1B
RAS	Retrovirus-associated DNA sequences
SDS	Sodium dodecyl sulfate
SHP-2	(SH2)-containing protein tyrosine phosphatase 2

SNP	Single nucleotide polymorphism
siRNA	Small interfering RNA
SOS	Salt Overly Sensitive
SOC	Super Optimal broth with Catabolite repression
TGN	Trans-Golgi network
TMD	Transmembrane domain
T2D	Type 2 diabetes
T2DM	Type 2 diabetes mellitus
WHO	World health organization
ZIP	Zrt/Irt-like, solute-linked carrier 39, SLC39
Zn2+	Zinc ion
ZnT	Solute-linked carrier 30, SLC30

GENERAL ABSTRACT

Zinc is a critical metal ion that has wide-ranging effects on cellular function. It is required for growth and development, immunity, and metabolism, and is therefore, vital for life. Disturbances in zinc homeostasis lead to various disease states including cancer, neurological disorders and diabetes. Recent studies have highlighted the dynamic role of zinc as an insulin mimetic and a cellular second messenger that controls many processes associated with insulin signalling and other downstream pathways that are amendable to glycaemic control. Therefore, mechanisms that contribute to the decompartmentalization of zinc and dysfunctional zinc transporter mechanisms, including zinc signalling are associated with metabolic disease.

As zinc cannot pass through biological membranes unassisted, cellular zinc storage, release and distribution are controlled by a family of proteins: zinc transporters and metallothioneins. Increasing evidence suggests that dysregulation of these proteins might act as key causative or promoting factors in several chronic pathologies such as insulin resistance and type 2 diabetes. Of these, emerging research has highlighted a role for several zinc transporters in the initiation of zinc signalling events in cells that lead to metabolic processes associated with maintaining insulin sensitivity and thus glycaemic homeostasis. One principle zinc transporter, Zip7 is emerging as a 'gate-keeper' of zinc flux from intracellular organelles including the Golgi apparatus and endoplasmic reticulum. Recent studies have identified that this transporter facilitates the mobilisation of intracellular zinc flux into the cytosol and the subsequent zinc-mediated activation of cell signalling molecules involved in glucose homeostasis. However, the mechanisms whereby zinc and Zip7 achieve glucose control is not known.

The present study evaluated the insulin-like effects of zinc on cell signalling pathways associated with controlling glucose homeostasis. Initially, key molecules involved in controlling glucose metabolism including tyrosine, PRSA40, Akt, ERK1/2, SHP-2, GSK-3β and p38, and the physiological response of glucose oxidation were analysed in the presence of zinc treatment in mouse and human skeletal muscle cells. Insulin and zinc treatment independently led to the phosphorylation of these proteins over a 60-minute time course in both mouse and human skeletal muscle cells and was concomitant with an increase in glucose oxidation. Similarly, utilising a commercially available protein array that contains several key proteins implicated in insulin signalling pathways we identified that zinc could active the

phosphorylation of p38, ERK1/2 and GSK-3β in human and ERK1/2 and GSK-3β in mouse skeletal muscle cells.

Glucose oxidation assays were performed on skeletal muscle cells treated with insulin, zinc, or a combination of both and resulted in a significant induction of glucose consumption in mouse and human skeletal muscle cells when treated with zinc alone. Insulin, as expected, increased glucose oxidation in mouse and human skeletal muscle cells, however the combination of zinc and insulin did not augment glucose consumption in these cells. Given that we did not observe an additive effect of insulin and zinc treatment together on glucose oxidation, we sought to determine whether a functional insulin receptor is required to facilitate zinc activation of pAkt. We utilised mouse C2C12 skeletal muscle cells treated with an insulin receptor tyrosine kinase inhibitor (HNMPA-(AM)3) in the presence of insulin or zinc. We observed that HNMPA-(AM)3 was sufficient to inhibit insulin-induced pAkt. Similarly, we identified that HNMPA-(AM)3 inhibited zinc-induced pAkt and suggests that zinc potentially acts through the insulin signalling pathway.

Previous studies have identified that Zip7 controls cell signalling pathways associated with glycaemic control in skeletal muscle cells. It was suggested that dysfunctional cell signalling processes in disease states such as insulin resistance and type 2 diabetes might be due to aberrant Zip7 expression and/or function. Accordingly, we initially set out to determine the role of Zip7 in an insulin-resistant cell culture model. Initially we tested the ability of glucose to regulate Zip7 protein levels. We found that 25 mM of glucose treatment of C2C12 mouse skeletal muscle cells significantly increased Zip7 protein levels. We also determined that Zip7 (and Glut4) were significantly reduced in insulin resistant skeletal muscle cells through inhibition of insulin receptor signalling and palmitate-induced insulin resistance. These studies suggest that Zip7 plays an important role in maintaining cell signalling processes associated with glucose control. We next focused our studies to determine if the expression of Zip7 changed in a mouse model of obesity. We found that Zip7 and the glucose transporter, Glut4 was reduced in obese mice fed a high fat diet.

From the above studies we established a Zip7 overexpression cell culture model to test the ability of this transporter to regulate genes implicated in glucose homeostasis. These studies were based on previous work which identified that a reduction in Zip7 in skeletal muscle cells led to several changes in genes associated with insulin signalling and glycaemic control.

Accordingly, utilising an insulin signalling pathway array that contained 84 genes involved in insulin signalling, we identified that overexpression of Zip7 in mouse C2C12 skeletal muscle cells led to significant changes in Akt3, Dok2, Fos, Hras, Kras, Nos2, Pck2, and Pparg.

In these studies, we demonstrated that zinc acts as an insulin mimetic, activating key molecules implicated in cell signalling to maintain glucose homeostasis in mouse and human skeletal muscle cells. Similarly, these studies demonstrated that Zip7 is involved in processes associated with insulin signalling and glucose control in skeletal muscle. This study also raises the possibility that zinc transporters could provide novel utility to be targeted experimentally and in a clinical setting to treat patients with insulin resistance and thus introduce a new class of drug target with utility for diabetes pharmacotherapy.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Background.

Type 2 diabetes mellitus (T2DM) is a chronic, progressive, and inadequately understood metabolic disease that is linked to several serious health problems, including cardiovascular disease, blindness, kidney failure, cognitive decline, and premature death (1). The number of people with diabetes (those having a fasting plasma glucose value of greater than or equal to 7.0 mmol/L or on medication for diabetes/raised blood glucose) has steadily risen over the past few decades, due to population growth, an increase in the average age of the population, and the rise in prevalence of diabetes with increasing age (2). Worldwide, the number of people with diabetes has substantially increased between 1980 and 2014, rising from 108 million to current number that are around four times higher. World health organization (WHO) estimates that, globally, 422 million adults aged over 18 years were living with diabetes in 2014 (3). The largest numbers of people with diabetes were estimated for the WHO South-East Asia and Western Pacific Regions (which includes Australia), accounting for approximately half the diabetes cases in the world. Also, the increase in the percentage of total deaths attributable to high blood glucose for adults aged 20-69 years, for years 2000 and 2012 was highest in the Western Pacific Region, where the total number of deaths attributable to high blood glucose during this period increased from 490,000 to 944,000 (2, 4).

A key characteristic of T2DM is insulin resistance (IR), a pathophysiological condition associated with disrupted biological responses to endogenous insulin which contributes to compromised glucose homeostasis specifically in liver, adipose tissue and skeletal muscle (5). Several years before being diagnosed with T2DM, a patient typically presents with IR. A leading concern for individuals with IR is compromised insulin secretion and function due to the chronic and progressive failure of pancreatic β-cell function (6). Therefore, there exists a "window of opportunity" to understand prevention strategies to halt or lessen the disease progression. This would have an enormous health impact for our society and for individuals with this disorder. Although T2DM is largely predictable through anthropometric, lifestyle and clinical factors, the molecular mechanisms and metabolic pathways that underpin the progression from normal glycaemia to IR and subsequent T2DM are inconclusive. Moreover, due to the lack of knowledge about the pleiotropic effects that medications have on specific molecular targets, treatments for IR have not progressed significantly during the last few decades (7). Therefore, finding strategies to discover new molecular targets that increase the efficacy and safety of therapeutic options for IR and T2DM are critical.

In this context, research on the molecular mechanisms and metabolic processes associated with IR and T2DM has revealed a biochemical and physiological role of zinc in the pathophysiology of these disorders (8). The suggestion that zinc deficiency and T2DM might be linked and that nutritional zinc supplementation could be efficacious to prevent this condition is appealing. It appears in some patients that zinc supplementation can improve the efficacy of oral hypoglycaemic agents by decreasing blood glucose, triglycerides and inflammation (9). However, in most cases of zinc-related cellular dysfunction, the defect appears to be associated with dysregulation of the proteins involved in controlling cellular zinc concentrations and the subsequent decompartmentalisation of zinc in subcellular organelles rather than one of generalised zinc deficiency (8). Accordingly, the research focus is shifting to the importance of zinc and the zinc transporter proteins that transport the metal ion as possible therapeutic targets for disease states associated with dysfunctional metabolism, including IR and T2DM. Several studies have determined a significant role for zinc as an insulin 'mimetic' in modulating cell signalling pathways involved in glucose homeostasis, and therefore may have therapeutic implications for IR and T2DM (10-12). Zinc is at the 'nexus' of controlling cell signalling molecules amendable to glucose homeostasis. However, the link between IR and the zinc modulation of proteins implicated in glucose homeostasis is unsubstantiated. As such, it is important to delineate the molecular mechanisms on how zinc activates cellular enzymes responsible for controlling the insulin-zinc signalling 'axis'. Importantly, the assessment of this 'axis' could lead to novel therapeutic strategies and interventions that are amendable to preclinical trials in controlling glucose homeostasis in IR and T2DM.

1.2. Insulin signalling and insulin resistance.

Insulin is a potent anabolic hormone which regulates a wide variety of biological processes in skeletal muscle including glucose uptake, glycogen synthesis, glucose transport, protein synthesis, and gene expression (13). The insulin receptor is a heterotetrameric protein consisting of two alpha-subunits localised to the extracellular space, and two transmembrane-bound beta-subunits connected by disulfide bonds (14). Insulin signalling involves a cascade of events initiated by insulin binding to the alpha-subunits of its receptor (15). This causes autophosphorylation of specific tyrosine residues in the insulin receptor tyrosine kinase domain of the intracellular part of the beta-subunit, and subsequent recruitment, binding and tyrosine phosphorylation of the insulin receptor substrate1 (IRS1), which mediates an association with phosphoinositide 3-kinase (PI3K) (14).

The next critical step in insulin signalling is the activation of Akt (protein kinase B), which is a serine/threonine kinase located downstream of PI3K (14). Akt serves as a highly regulated point for downstream signalling to 1) transport glucose through the glucose transporter GLUT4 (in skeletal muscle), 2) to synthesize glycogen through glycogen synthase kinase 3 (GSK3) and glycogen synthase (GS) (16), also 3) to synthesize protein through the mammalian target of rapamycin (mTOR) pathway (17). Akt mediates GLUT4 translocation in response to insulin and promotes glucose transportation into the cells. In the basal state, the majority of GLUT4 protein is localised intracellularly. Insulin stimulates glucose transport by promoting GLUT4 exocytosis and promoting the tethering, docking, and thus fusion of GLUT4 vesicles to the outer plasma membrane and subsequent uptake and import of extracellular glucose into the cell (18).

Extracellular signal-regulated protein kinase1/2 (ERK1/2), SHP-2 and Protein-Rich Akt Substrate of 40 kDa (PRAS40) are known as Akt mediators in insulin signalling pathway. SHP-2 is a protein tyrosine phosphatase involved in insulin signalling, and can bind to IRS1 in response to insulin to mediate receptor tyrosine kinase- signalling (19). PRAS40 is also among the most prominent Akt substrate that is phosphorylated in response to insulin in eukaryotes (20). PRAS40 also decreases mTOR expression which is a negative regulator of IRS1 in skeletal muscle, and mediates an increase in insulin-stimulated Akt phosphorylation and glucose uptake (20). **Figure 1.1** below shows an overview of the insulin signalling pathway.

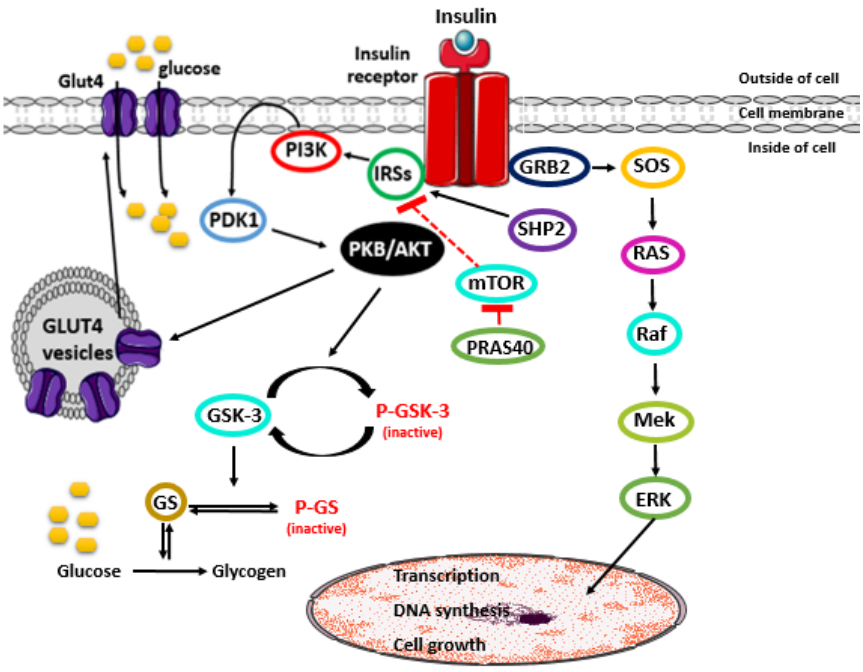

Figure 1.1. Schematic diagram of the insulin signalling pathway. Insulin mediates its action through binding to the insulin receptor, activation of insulin receptor substrates (IRSs) and PI3K, and induction of Akt phosphorylation. This mediates the mobilisation and translocation of intracellular Glut4 molecules to the outer plasma membrane surface and initiation of glucose uptake into fat and muscle cells. Other components include the activation of the SOS/RAS pathway leading to increased ERK phosphorylation and transcriptional activation of growth promoting factors. (GLUT4: Glucose transporter 4, PI3K: Phosphoinositide 3 kinase, PDK1: Pyruvate Dehydrogenase Kinase 1, PKB: Protein kinase B, GRB2: Growth factor receptor-bound protein 2, SHP2: (SH2)-containing protein tyrosine phosphatase 2, mTOR: Mammalian target of rapamycin, PRAS40: Proline-rich AKT substrate of 40 kDa, SOS: Salt Overly Sensitive, Mek: Mitogen-activated protein kinase kinase, ERK: Extracellular signal-regulated protein kinase, GSK-3: Glycogen synthesis kinase-3, GS: Glycogen synthase). Figure was produced using Smart Servier Medical Art, http://smart.servier.com.

Promotion of glucose transport and glycogen synthesis are key biological actions of insulin in skeletal muscle. Defects in these actions appear to be major determinants of skeletal muscle IR. IR can be defined as an impaired biological response to either endogenous or exogenous insulin. This is an extremely common pathophysiological condition in which patients present with diminished insulin sensitivity and thus reduced insulin-stimulate glucose uptake and glycogen synthesis, particularly in skeletal muscle and liver (21). This has major implications for the individual as they are unable to obtain the required energy from glucose to sustain cellular metabolic processes. IR is of major global concern as it is associated with the development of several disorders and disease states including T2DM, hyperglycaemia, obesity, polycystic ovary syndrome (PCOS), liver cirrhosis (13) atherosclerosis, hypertension and stroke (22).

Although several somatic cell lines express insulin receptors, the role of insulin in glucose homeostasis is characterized by its direct effects on skeletal muscle, liver, and white adipocyte tissues which they perform different roles in metabolic homeostasis, necessitating tissue-specific insulin signal transduction pathways (23). For instance, insulin promote glucose consumption and storage in skeletal muscle cells by improving glucose transport and glycogen synthesis (24). In liver, insulin plays an important role to increase glycogen synthesis and lipogenic gene expression, and to decrease the expression of gluconeogenic gene (25). In white

adipocytes, insulin decreases lipolysis and increases glucose transport and lipogenesis. Despite these diverse roles, the proximal components involved in insulin signal transduction are very similar in different types of insulin responsive cell lines (26).

The major function of insulin in skeletal muscle is to increase cellular glucose uptake through the translocation of Glut4. Insulin-stimulated skeletal muscle glucose uptake is extremely susceptible to IR and is indeed a key component of T2DM (26). Because skeletal muscle tissue is the predominant site of insulin-mediated glucose disposal, muscle IR has a huge effect on the whole body glucose turnover (26, 27). Insulin stimulation of glycogen synthesis and glycolysis both require intact insulin-stimulated glucose uptake to replenish substrate availability, so these effects also become resistant to insulin action (21). In patients with T2DM, the translocation of glucose into the cell is the major rate-controlling step responsible for the reduction in insulin-stimulated muscle glycogen synthesis, which is defective in IR condition (26).

Skeletal muscle IR is observable to defects at the most proximal levels of insulin signalling pathway including insulin receptor, IRS1, PI3K, and AKT activity. Defects in insulin receptor kinase activity is also suggested by reduced IRS1 tyrosine phosphorylation observed in insulin-resistant skeletal muscle cells (28). Blunted insulin stimulation of IRS1-associated PI3K activity is also reproducibly observed in skeletal muscle IR. Interestingly, skeletal muscle of patients with T2DM does not develop IR to mitogenic signalling through Mitogen-activated protein kinase (MAPK) (28). Although impaired insulin activation of other distal effectors, including AKT, is often seen in skeletal muscle IR, the simultaneous presence of proximal insulin signalling defects makes it difficult to realise whether these distal defects have an independent origin or are secondary to proximal defects (26, 29).

Treatments for IR and T2DM have not advanced significantly in the last few decades due to the insufficient knowledge about the pleiotropic effects that many diabetic drugs have on molecular targets. Accordingly, this leads into a suggested role for zinc in the treatment of IR and T2DM through understanding the mechanisms of action of zinc protein transporters (10).

1.3. Zinc, zinc transporter proteins and insulin signalling.

It has long been a consensus that a well-balanced diet, exercise and diabetic agents are cornerstones for the prevention and treatment of IR and T2DM. However, reducing the decline and eventual failure of functioning pancreatic beta-cells is of paramount importance in preventing T2DM and halting disease progression in affected patients (30). The increased global prevalence of T2DM and the progressive decline in metabolic control in patients are clear demonstrations that present therapeutic strategies are somewhat inadequate. Therefore, there are pressing requirements for anti-diabetic agents that target metabolic processes before the development of beta-cell destruction and T2DM progression.

Recently, research on type 2 diabetes has revealed an exciting role for zinc as a dietary supplementation in diseases associated with abnormal cellular signalling and metabolism such as IR (31). Zinc is an interesting candidate metal ion since it is a component of insulin crystals. Before insulin crystallisation in pancreatic β-cells, and subsequently delivery into the blood circulation, this hormone is packaged as Zn^{2+} coordinated hexamers to increase the stabilisation of insulin monomers (31, 32). This suggests that disruptions in zinc homeostasis would have severe consequences on the packaging, storage and secretion of insulin and therefore contribute to glucose intolerance and subsequent failure of beta-cell function.

In the human body there is approximately two to four grams of total zinc making it the most abundant trace element in tissue next to iron (approximately four grams which is localised primarily in blood) (12). At the cellular level, 30-40% of total cellular zinc is found in the nucleus, 50% in the cytosol and organelles (such as the endoplasmic reticulum), and the remainder is found in plasma membranes. Zinc is essentially bound to macromolecules within these compartments including zinc proteins/enzymes, lipids, and DNA/RNA. In fact, research of the human genome has established that approximately 10% of the proteome consists of potential zinc binding proteins, now identified as the "zincome" (12). Accordingly, the compartmentalisation, availability, transport and re-distribution of zinc must be tightly controlled to maintain a balance between health and disease states (11). Moreover, since zinc cannot pass through biological membranes unassisted, several proteins including metallothioneins and zinc transporters are involved in the intrinsic control of intracellular/extracellular zinc concentrations in mammals (8, 33, 34).

Metallothionein (MT) are small cysteine-rich proteins which are dedicated to controlling zinc homeostasis in humans and other mammals (35). MTs are redox-active zinc proteins that buffer and translocate zinc within the cytosol depending on the availability of cellular zinc. MTs participate in cellular metal muffling; a process that contributes to the cellular distribution and compartmentalisation of zinc to maintain zinc homeostasis (36).

The second group of factors involved in controlling zinc movement, and thus zinc concentrations in cells, are zinc transporter proteins. Two families exist; the ZIP proteins (Zrt/Irt-like, solute-linked carrier 39, SLC39) and the ZnT proteins (solute-linked carrier 30, SLC30). ZIP and ZnT family members facilitate zinc transport into the cytosol and out of the cytosol, respectively (37, 38). The balance between ZIP and ZnT transporters regulates cytosolic and intra-organelle zinc levels (39). A similar protein structure is observed between the same family members of these transporters (37) and suggests that they might have overlapping fiction or some protein redundancy, although this still needs to be established.

Given the role of zinc as an insulin "memetic" and a modulator of several cell signalling events leading to glucose utilisation, and the specificity of zinc transporter proteins in maintaining cellular zinc homeostasis, it is likely that aberrant expression of these transporters and/or zinc departmentalisation will have deleterious effects on cellular viability.

1.4. Thesis Overview

The following Chapters outlined below provide a "snap shot" of the subsequent larger Chapters to better understand the role of zinc in cellular processes associated with glycaemic control in skeletal muscle and to highlight its potential mechanisms of action. It should also be highlighted that this thesis is a combination of traditional thesis text (Chapters 1, 4, and 6) and peer-reviewed published articles (Chapters 2, 3 and 5).

1.4.1. Chapter 2: Norouzi, Shaghayegh, Adulcikas, John, Sohal, Sukhwinder Singh and Myers, Stephen (2017), Zinc transporters and insulin resistance: therapeutic implications for type 2 diabetes and metabolic disease. Published in the *Journal of Biomedical Science*, 2017, 24:87; DOI: 10.1186/s12929-017-0394-0.

This Chapter examined in detail the role of zinc transporter proteins and the mechanisms involved in the decompartmentalisation of zinc and dysfunctional zinc signalling associated with metabolic disease including T2DM. The Chapter introduces the reader to the development and pathophysiology of T2DM. It outlines the importance of current drug therapy for the treatment of T2DM and the need to develop novel therapeutic targets that have greater efficacy and safety. The Chapter then discusses zinc and its role as an essential trace metal ion and the compartmentalisation of zinc in various cellular organelles. This is highly significant because zinc is tightly controlled and decompartmentalisation of zinc is associated with various disease states including T2DM (10). The Chapter then discusses the zinc transporter family in detail with a specific focus on their roles in cellular signalling and insulin resistance. Of the zinc transporters some detail on the role of these proteins in T2DM is given where possible. However, extrapolation of zinc transporters involved in other metabolic diseases and their role in cell signalling processes associated with cellular homeostasis provides greater clarity to their potential function. Finally, a role for the zinc transporter protein ZnT8 is discussed as a significant representative of this family of proteins in maintaining insulin storage, secretion, and action and how understanding the mechanisms of this transporter (and others) could help elucidate novel therapeutic options for the treatment of early diabetic symptoms and T2DM (10).

Research has revealed an important role for zinc and zinc transporters in diseases associated with abnormal cellular signalling and metabolism (11). The zinc transporter proteins are unique as they can release zinc into the cytosol from intracellular organelles to affect/direct cell signalling events (39). Accordingly, zinc transporters have emerged as potential therapeutic targets associated with dysfunctional disease states of metabolism. Emerging research has also shown that zinc can initiate zinc-mediated signalling events in cells that lead to metabolic processes associated with insulin secretion and glucose homeostasis (39). For example, ZnT8, a member of the ZnT zinc transporter family, is critical for the compartmentalisation, structure and secretion of insulin in beta-cells of the pancreas (10). It has been shown that ZnT8 plays an important role in the development of T2DM in humans. In pancreatic β-cells, ZnT8 is highly expressed in the insulin secretory vesicles close to the cell membrane (37). Cytoplasmic zinc is translocated into the insulin-containing vesicles *via* ZnT8 to be utilised for insulin crystallisation. Mice with pancreatic ZnT8 knockout have defects in the processing, crystallisation and secretion of insulin from pancreatic beta-cells (37). These data clearly show that ZnT8 is essential for proper beta-cell function and insulin homeostasis. Moreover, a single-

nucleotide polymorphism (SNP) at amino acid position 325 of the ZnT8 transporter (rs13266634) causes susceptibility to diabetes in humans (37).

ZnT7, another member of the ZnT transporter family, plays an important role in the translocation of cytosolic zinc into the Golgi apparatus and ZnT7-containing granules. In insulin secreting β-cells, ZnT7 overexpression causes an increase in insulin synthesis and secretion (40). It has also been shown that total body zinc concentration is decreased in Znt7 knock-out mice compared to the wild-type controls (37). In addition, Znt7 knock-out mice also have higher blood glucose levels two hours after an oral glucose administration, indicating that the Znt7-null mutation may affect glucose homeostasis (37). In fact, ZnT7 affects glucose homeostasis and pathogenesis of diabetes through regulating the insulin signalling pathway in insulin sensitive peripheral tissues such as skeletal muscle (37).

ZIP6 and ZIP7, members of the ZIP transporter family, may also play a role in pancreatic insulin homeostasis *via* changes in the pool of available zinc in the cytosol and/or subcellular organelles. Knock-down studies in mouse islet cells that reduced the levels of ZIP6 and ZIP7 resulted in severe oxidative stress and a significant reduction in glucose-stimulated zinc uptake (39). These authors speculate that the compromised expression of ZIP6 and ZIP7 potentially disrupts zinc homeostasis and produces defects in beta cell function and insulin secretion that may initiate the development of diabetes.

Research has also identified ZIP7 is expressed exclusively in the endoplasmic reticulum and the Golgi apparatus to act as the "gate-keeper" of zinc release from these subcellular organelles where it is involved in regulating cell signalling and metabolism (41). A reduction in the expression of ZIP7 *via* small interfering RNA (siRNA) in skeletal muscle cells resulted in a significant reduction in genes and proteins involved in glucose metabolism, including the insulin receptor, IRS1 and IRS2, Akt phosphorylation, glucose transporter Glut4, and glycogen branching enzyme (42). These data suggest that ZIP7 is involved in the cellular release of zinc that acts on metabolic pathways that lead to glycaemic control in skeletal muscle (42). However, the potential mechanism by which zinc and Zip7 can improve glycaemic control is not understood. In Chapter 3, 4 and 5, this is evaluated to determine the potential roles of zinc and Zip7 on insulin signalling and glucose metabolism in skeletal muscle.

1.4.2. Chapter 3: Norouzi, Shaghayegh, Adulcikas, John, Sohal, Sukhwinder Singh and Myers, Stephen (2018). Zinc stimulates glucose oxidation and glycemic control by modulating the insulin signalling pathway in human and mouse skeletal muscle cells. Published in *PLOS ONE*, 10.1371/journal.pone.0191727.

This Chapter discusses the important role of zinc in activating the insulin-zinc axis in human and mouse skeletal muscle cells. The Chapter demonstrates the role of zinc as an insulin mimetic in activating key molecules involved in insulin signalling and glucose homeostasis including phospho-tyrosine, PRSA40, Akt, ERK1/2, SHP-2, GSK-3β and p38 and the concomitant increase in glucose oxidation in human and mouse skeletal muscle cells (43). It also identified that the phosphorylation of Akt, ERK1/2, SHP, and tyrosine could be induced by 20 μM of zinc in mouse C2C12 and human skeletal muscle cells. These studies were confirmed by treatment of the skeletal muscle cells with 10 nM of insulin which also led to the phosphorylation of these molecules. Insulin served as a control as its signalling action on these molecules is well-established (43). Similarly, this paper showed that insulin and zinc could activate PRAS40, GSK-3β and p38 in a mouse and human protein array that contained several proteins implicated in the insulin signalling pathway (43). These studies are the first to comprehensively confirm the activation of several insulin signalling molecules by zinc in skeletal muscle and further highlights zinc's insulin mimetic role in the zinc-insulin axis.

Chapter 3 also describes the effect of glucose consumption through zinc-mediated mechanisms in human and mouse skeletal muscle cells. It was identified that in the presence of zinc, glucose oxidation was enhanced significantly. These studies were supported by a similar effect with insulin treatment where it is well established that insulin increases glucose oxidation in skeletal muscle cells (43).

While it is unclear of the mechanisms whereby zinc activates glucose oxidation, some preliminary data in this Chapter suggests that Akt is involved. To address this, we sought to determine if a functional insulin receptor was required for the zinc-mediated action on Akt. Inhibition of the insulin receptor with a specific antagonist HNMPA-(AM)3 completely

abolished insulin-mediated phosphorylation of Akt as expected (43). Similarly, inhibition of the insulin receptor eliminated zinc-mediated activation of Akt. These studies therefore suggest that zinc-mediated activation of Akt requires a functional insulin receptor (43). Thus, zinc independently activates proteins involved in the insulin signalling pathway and both zinc and insulin play critical roles in maintaining glucose homeostasis in mouse and human skeletal muscle cells.

As mentioned above, we have shown that zinc could enhance glucose oxidation in skeletal muscle cells (43). We hypothesised that the increased glucose oxidation by zinc is due to the increased glucose transporter protein (Glut4) translocation to the cell membrane and consequent increased glucose uptake by Glut4 transporters. This was investigated in Chapter 4 outlined below.

1.4.3. Chapter 4 investigated the effects of zinc on Glut4 mobilisation and glucose homeostasis. It is well stablished that insulin induces translocation of the glucose transporter Glut4 from intracellular storage compartment to the plasma membrane in skeletal muscle (44). Glut4 in muscle and adipose tissue is indispensable for glucose homeostasis. Animal studies that show a tissue-specific (adipose and skeletal muscle) deletion of Glut4 compromises glucose homeostasis and initiates insulin resistance (45). This is in contrast to the deletion of insulin-signalling components from these tissues having minimal effect on compromising glucose homeostasis (45). These studies highlight the extreme importance of a functional Glut4 despite a compromised insulin signalling axis. Moreover, muscle-specific Glut4–knockout mice develop severe hyperglycaemia, glucose intolerance, and insulin resistance by eight weeks of age (46). It remains unclear whether zinc has a regulatory role in mobilising Glut4 in skeletal muscle. Accordingly, these studies presented in Chapter 4 were designed to address the zinc-mediated activity of Glut4 in C2C12 skeletal muscle cells. Initially we measured the protein levels of Glut4 in the presence of zinc for a period of 16 hours and observed that zinc increased Glut4 protein expression after two hours of treatment in mouse C2C12 skeletal muscle cells. Then, total membrane and cytoplasm fractions were prepared to measure Glut4 levels in cells treated with zinc and insulin (as a control). Glut4 transporters were highly abundant in the membrane of the skeletal myocytes treated with insulin for 15 minutes. We did not detect any changes in the Glut4 membrane abundancy in the skeletal myocytes treated with

zinc for 15 minutes compared to the control (untreated cells). In fact, it appeared that the experimental procedures might have contributed to this unexpected result. This is outlined in detail in Chapter 4.

We also investigated mobilisation and translocation of Glut4 from intracellular vesicles to the outer plasma membrane in C2C12 cells treated with zinc or insulin by confocal microscopy. We utilised two strategies to test this, 1) measurement of endogenous Glut4, and 2) measurement of exogenous Glut4 bound to Green Fluorescent Protein (Glut4-GFP). According to the results, insulin or zinc treatment did not significantly affect mobilisation and translocation of Glut4 to the skeletal muscle outer plasma membrane surface from intracellular stores in the presence of insulin or zinc. Several reasons why these experiments did not result in translocation of Glut4 for are given in Chapter 4. Of these, several research articles suggest that C2C12 cells have diminished responses to insulin and reduced Glut4 machinery to enable efficient translocation (43). However, this is not congruent with the data from our recent publications and others that show insulin can activate pAkt and lead to glucose uptake in these cell lines (43, 47). In addition, it is not clear why zinc treatment did not initiate the translocation of Glut4. Evidence to possibly support this result are given in Chapter 4.

1.4.4. Chapter 5. Norouzi, S. Adulcikas, J. Henstridge, D. C. Sonda, S. Sohal, S. S. and Myers, S. (2019). The Zinc Transporter Zip7 Is Downregulated in Skeletal Muscle of Insulin-Resistant Cells and in Mice Fed a High-Fat Diet, Published in Cells, 8: DOI: 10.3390/cells8070663.

Given we have identified several lines of evidence showing zinc can activate several cell signalling molecules involved in glucose uptake, we turned our attention to the "gate-keeper" of zinc flux, Zip7. It has previously been shown that Zip7 ablation resulted in reduced Glut4 levels as well as reduced insulin-stimulated glycogen synthesis in mouse skeletal muscle cells (42). These data suggest that Zip7 is involved in the cellular release of zinc that acts on metabolic pathways that lead to glycaemic control in skeletal muscle.

To further investigate the role of Zip7 on insulin signalling and glucose metabolism, in Chapter 5 we examined the effect of hyperglycaemia in mouse skeletal muscle cells treated with glucose and measured Zip7 expression. It was identified that treatment of mouse skeletal muscle cells with 25 mM glucose robustly upregulated Zip7 protein. Given that Zip7 protein expression

could be regulated by glucose, several studies were performed to recapitulate a compromised glucose state in mouse skeletal muscle cells. Accordingly, two experimental procedures were utilised to create an insulin resistant state in skeletal muscle cells: 1) an inhibitor of the insulin receptor HNMPA-(AM)3, and 2) fatty acid (palmitate) treatment. It was identified that Zip7 (and Glut4) were significantly reduced in insulin resistant mouse skeletal muscle cells using the pharmacological inhibitor of the insulin receptor HNMPA-(AM)3 and palmitate. A significant decrease in the Akt phosphorylation and tyrosine expression was also observed. These studies were intriguing. Given Zip7 is down regulated in a classic insulin signalling pathway suggests that this transporter has the potential to modulate the cell signalling pathways induced by insulin. To this end, an overexpression Zip7 plasmid was transfected into mouse skeletal muscle cells and tested for its ability to regulate genes involved in the insulin signalling and glucose homeostasis. A significant overexpression of Zip7 mRNA and protein was observed in the skeletal muscle cells. Utilising a commercially available insulin signalling pathway array, we detected significant changes (overexpressions and downregulations) in several genes including Akt3, Dok2, Fos, Hras, Kras, Nos2, Pck2 and Pparg. These studies suggested that Zip7 plays a critical role in maintaining cell signalling processes associated with glucose control in mouse skeletal muscle.

Similarly, Zip7 is reduced in an insulin-resistant state when treated pharmacologically with fatty acids. This suggests that Zip7 could be regulated by changes in fatty acids, palmitate or a high fat diet (HFD). These results prompted additional studies in an *in vivo* obese mouse model. Accordingly, C57 black 6 mice were fed a diet of normal chow (NC) or HFD for 10 weeks. As expected on the HFD, body fat, body mass, and fat percentages were increased in the HFD versus the NC. Similarly, an oral glucose tolerance test revealed that the HFD had reduced glucose clearance compared to the NC controls. To determine the protein expression of Zip7 in NC versus HFD, western blots were performed. It was identified that the protein expression of both Zip7 and Glut4 were reduced in HFD compare to the NC controls. These data suggest that Zip7 plays a role in regulating insulin signalling pathways in skeletal muscle cells and these pathways are compromised when Zip7 is downregulated in an insulin resistant state.

1.5. Summary

To summarise, IR is a medical disorder that is associated with the development of T2DM. Zinc is a crucial component of insulin signalling and glucose metabolism and it may delay the onset

of T2DM among patients with IR. Zinc is able to activate insulin signalling molecules and increase glucose oxidation possibly independent of insulin in skeletal muscle cells. However, this still needs be confirmed. Atypical levels of zinc or aberrant compartmentalisation, transport and storage of this ion will have biological effects that could be amendable to clinical intervention. Zip7, as the gate keeper of zinc release from subcellular organelles, acts on metabolic pathways involved in glucose haemostasis by modulating insulin signalling molecules and is reduced in an insulin-resistant state and in mice fed a HFD. Further studies are required to clear the molecular mechanism by which zinc and Zip7 contribute in insulin signalling and glycaemic control.

1.6. Hypothesis and aims

While studies elucidating the role of zinc and zinc transporters in various disease states are emerging, their role in activating cell signalling molecules associated with insulin sensitivity and glucose metabolism is not well-established. Accordingly, the overall hypothesis of this thesis is:

Zinc and the zinc transporter Zip7 regulate insulin sensitivity in skeletal muscle cells

Skeletal muscle cells respond to insulin and previous studies have shown that insulin is required to activate cell signalling molecules such as the phosphorylation of Akt that are essential for driving insulin-specific glycaemic control (42). To determine if zinc is involved in signalling events independent, or dependent on insulin activity, the following Aims were performed.

Aim 1: To determine the action of insulin and zinc on insulin-mediated signalling in skeletal muscle cells.

IR is a precursor to T2DM and studies addressing the molecular mechanisms of IR are critical to develop novel therapies to better manage this disorder before the development of T2DM. To determine if IR affects zinc-dependent cell signalling events, the following Aim 2 was proposed.

Aim 2: To create insulin-resistant skeletal muscle cells and determine the effect of zinc on insulin signalling.

The zinc transporter ZIP7 is implicated in the "gated" release of zinc into the cytosol where it can act on cell signalling events that lead to metabolic processes associated with cell viability (41). The following Aim 3 was proposed to address the role of Zip7-mediated activation of insulin-dependent cell signalling pathways.

Aim 3: To determine the role of Zip7-mediated cell signalling events controlling glucose metabolism in insulin resistant skeletal muscle cells and obese mice.

References

1. Bellamy L, Casas J-P, Hingorani AD, Williams D. Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. The Lancet. 2009;373(9677):1773-9.
2. Collaboration NRF. Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4· 4 million participants. The Lancet. 2016;387(10027):1513-30.
3. Smolen J, Burmester G, Combeet B. NCD Risk Factor Collaboration (NCD-RisC). Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4· 4 million participants. Lancet2016; 387: 1513–30—In this Article, Catherine Pelletier.
4. Organization WH. Tracking universal health coverage: first global monitoring report: World Health Organization; 2015.
5. Abdul-Ghani MA, DeFronzo RA. Pathogenesis of Insulin Resistance in Skeletal Muscle. Journal of Biomedicine and Biotechnology, doi:10115/2010/476279. 2010.
6. Pajvani UB, Accili D. The new biology of diabetes. Diabetologia. 2015;58(11):2459-68.
7. Pajvani UB, Accili D. The new biology of diabetes. Diabetologia. 2015;58(11):2459-68.
8. Mocchegiani E, Giacconi R, Malavolta M. Zinc signalling and subcellular distribution: emerging targets in type 2 diabetes. Trends in Molecular Medicine. 2008;14(10):419-28.
9. Jayawardena R, Ranasinghe P, Galappatthy P, Malkanthi R, Constantine G, Katulanda P. Effects of zinc supplementation on diabetes mellitus: a systematic review and meta-analysis. Diabetology & Metabolic Syndrome. 2012;4(1):13.
10. Norouzi S, Adulcikas J, Sohal SS, Myers S. Zinc transporters and insulin resistance: therapeutic implications for type 2 diabetes and metabolic disease. Journal of biomedical science. 2017;24(1):87.
11. Myers SA, Nield A, Myers M. Zinc transporters, mechanisms of action and therapeutic utility: implications for type 2 diabetes mellitus. Journal of nutrition and metabolism. 2012;2012.
12. Myers SA, Nield A. Zinc, Zinc Transporters and Type 2 Diabetes. 2014.
13. Højlund K. Metabolism and insulin signaling in common metabolic disorders and inherited insulin resistance. Danish medical journal. 2014;61(7):B4890-B.
14. Gokhale KM. Role of glycogen synthase kinase (GSK-3) in type-2 diabetes and GSK-3 inhibitors as potential anti-diabetics. International Journal of Pharmaceutical and Phytopharmacological Research. 2014;3(3).
15. Gokhale KM, Tilakb BP. International Journal of Pharmaceutical and Phytopharmacological Research (eIJPPR).
16. Gokhale KM. Role of glycogen synthase kinase (GSK-3) in type-2 diabetes and GSK-3 inhibitors as potential anti-diabetics. International Journal of Pharmaceutical and Phytopharmacological Research. 2013;3(3).
17. Copps K, White M. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia. 2012;55(10):2565-82.
18. Kramer HF, Witczak CA, Fujii N, Jessen N, Taylor EB, Arnolds DE, et al. Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle. Diabetes. 2006;55(7):2067-76.
19. Noguchi T, Matozaki T, Horita K, Fujioka Y, Kasuga M. Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation. Molecular and cellular biology. 1994;14(10):6674-82.
20. Wiza C, Chadt A, Blumensatt M, Kanzleiter T, Herzfeld De Wiza D, Horrighs A, et al. Over-expression of PRAS40 enhances insulin sensitivity in skeletal muscle. Archives of physiology and biochemistry. 2014;120(2):64-72.
21. DeFronzo RA, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes care. 2009;32(suppl 2):S157-S63.
22. Taghibiglou C, Rashid-Kolvear F, Van Iderstine SC, Le-Tien H, Fantus IG, Lewis GF, et al. Hepatic very low density lipoprotein-ApoB overproduction is associated with attenuated hepatic insulin

signaling and overexpression of protein-tyrosine phosphatase 1B in a fructose-fed hamster model of insulin resistance. Journal of Biological Chemistry. 2002;277(1):793-803.
23. Perseghin G, Calori G, Lattuada G, Ragogna F, Dugnani E, Garancini MP, et al. Insulin resistance/hyperinsulinemia and cancer mortality: the Cremona study at the 15th year of follow-up. Acta diabetologica. 2012;49(6):421-8.
24. Vila G, Jørgensen JOL, Luger A, Stalla GK. Insulin resistance in patients with acromegaly. Frontiers in endocrinology. 2019;10:509.
25. Sharabi K, Tavares CD, Puigserver P. Regulation of Hepatic Metabolism, Recent Advances, and Future Perspectives. Current diabetes reports. 2019;19(10):98.
26. Petersen MC, Shulman GI. Mechanisms of insulin action and insulin resistance. Physiological reviews. 2018;98(4):2133-223.
27. Kowalski GM, Bruce CR. The regulation of glucose metabolism: implications and considerations for the assessment of glucose homeostasis in rodents. Am J Physiol-Endocrinol Metab. 2014;307(10):E859-E71.
28. Cusi K, Maezono K, Osman A, Pendergrass M, Patti ME, Pratipanawatr T, et al. Insulin resistance differentially affects the PI 3-kinase–and MAP kinase–mediated signaling in human muscle. The Journal of clinical investigation. 2000;105(3):311-20.
29. Borisov N, Aksamitiene E, Kiyatkin A, Legewie S, Berkhout J, Maiwald T, et al. Systems-level interactions between insulin–EGF networks amplify mitogenic signaling. Molecular systems biology. 2009;5(1).
30. Ranasinghe P, Pigera S, Galappatthy P, Katulanda P, Constantine GR. Zinc and diabetes mellitus: understanding molecular mechanisms and clinical implications. Daru : journal of Faculty of Pharmacy, Tehran University of Medical Sciences. 2015;23:44.
31. Arvan P, Halban PA. Sorting ourselves out: seeking consensus on trafficking in the beta-cell. Traffic (Copenhagen, Denmark). 2004;5(1):53-61.
32. Rutter GA, Chabosseau P, Bellomo EA, Maret W, Mitchell RK, Hodson DJ, et al. Intracellular zinc in insulin secretion and action: a determinant of diabetes risk? Proceedings of the Nutrition Society. 2016;75(1):61-72.
33. Devirgiliis C, Zalewski PD, Perozzi G, Murgia C. Zinc fluxes and zinc transporter genes in chronic diseases. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2007;622(1–2):84-93.
34. Kambe T, Matsunaga M, Takeda TA. Understanding the Contribution of Zinc Transporters in the Function of the Early Secretory Pathway. International journal of molecular sciences. 2017;18(10).
35. Vašák M, Hasler DW. Metallothioneins: new functional and structural insights. Current opinion in chemical biology. 2000;4(2):177-83.
36. Maret W. Redox biochemistry of mammalian metallothioneins. JBIC Journal of Biological Inorganic Chemistry. 2011;16(7):1079-86.
37. Huang L, Kirschke CP, Lay Y-AE, Levy LB, Lamirande DE, Zhang PH. Znt7-null mice are more susceptible to diet-induced glucose intolerance and insulin resistance. Journal of Biological Chemistry. 2012;287(40):33883-96.
38. Kambe T. An overview of a wide range of functions of ZnT and Zip zinc transporters in the secretory pathway. Bioscience, Biotechnology, and Biochemistry. 2011;75(6):1036-43.
39. Liu Y, Batchuluun B, Ho L, Zhu D, Prentice KJ, Bhattacharjee A, et al. Characterization of Zinc Influx Transporters (ZIPs) in Pancreatic β Cells Roles in Regulating Cytosolic Zinc Homeostasis and Insulin Secretion. Journal of Biological Chemistry. 2015;290(30):18757-69.
40. Huang L, Yan M, Kirschke CP. Over-expression of ZnT7 increases insulin synthesis and secretion in pancreatic β-cells by promoting insulin gene transcription. Experimental cell research. 2010;316(16):2630-43.
41. Taylor KM, Hiscox S, Nicholson RI, Hogstrand C, Kille P. Protein kinase CK2 triggers cytosolic zinc signaling pathways by phosphorylation of zinc channel ZIP7. Science signaling. 2012;5(210):ra11-ra.

42. Myers SA, Nield A, Chew G-S, Myers MA. The zinc transporter, Slc39a7 (Zip7) is implicated in glycaemic control in skeletal muscle cells. PLoS One. 2013;8(11):e79316.
43. Norouzi S, Adulcikas J, Sohal SS, Myers S. Zinc stimulates glucose oxidation and glycemic control by modulating the insulin signaling pathway in human and mouse skeletal muscle cell lines. PLoS One. 2018;13(1):e0191727.
44. Ijuin T, Takenawa T. SKIP negatively regulates insulin-induced GLUT4 translocation and membrane ruffle formation. Molecular and cellular biology. 2003;23(4):1209-20.
45. Centner S. The Effects of Intermittent Fasting on Blood Insulin Levels and Insulin Sensitivity: A Literature Review.
46. Nedachi T, Kanzaki M. Regulation of glucose transporters by insulin and extracellular glucose in C2C12 myotubes. Am J Physiol-Endocrinol Metab. 2006;291(4):E817-E28.
47. Tang S, Le-Tien H, Goldstein BJ, Shin P, Lai R, Fantus IG. Decreased in situ insulin receptor dephosphorylation in hyperglycemia-induced insulin resistance in rat adipocytes. Diabetes. 2001;50(1):83-90.

CHAPTER 2

ZINC TRANSPORTERS AND INSULIN RESISTANCE: THERAPEUTIC IMPLICATIONS FOR TYPE 2 DIABETES AND METABOLIC DISEASE

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REVIEW Open Access

Zinc transporters and insulin resistance: therapeutic implications for type 2 diabetes and metabolic disease

Shaghayegh Norouzi, John Adulcikas, Sukhwinder Singh Sohal and Stephen Myers*

Abstract

Background: Zinc is a metal ion that is essential for growth and development, immunity, and metabolism, and therefore vital for life. Recent studies have highlighted zinc's dynamic role as an insulin mimetic and a cellular second messenger that controls many processes associated with insulin signaling and other downstream pathways that are amendable to glycemic control.

Main body: Mechanisms that contribute to the decompartmentalization of zinc and dysfunctional zinc transporter mechanisms, including zinc signaling are associated with metabolic disease, including type 2 diabetes. The actions of the proteins involved in the uptake, storage, compartmentalization and distribution of zinc in cells is under intense investigation. Of these, emerging research has highlighted a role for several zinc transporters in the initiation of zinc signaling events in cells that lead to metabolic processes associated with maintaining insulin sensitivity and thus glycemic homeostasis.

Conclusion: This raises the possibility that zinc transporters could provide novel utility to be targeted experimentally and in a clinical setting to treat patients with insulin resistance and thus introduce a new class of drug target with utility for diabetes pharmacotherapy.

Keywords: Zinc ions, Skeletal muscle, Cell signaling, Glycemic control

Background

Insulin resistance (IR) is a common pathophysiological condition in which patients present with reduced insulin sensitivity and thus glucose intolerance, particularly in liver, adipose tissue and skeletal muscle [1]. This has significant implications for the patient, as they are unable to obtain to process the required energy from glucose to maintain cellular metabolic processes. IR is of major global concern as it is well-established as underpinning many chronic health conditions including type 2 diabetes mellitus (T2DM), obesity, cardiovascular disease polycystic ovary syndrome (PCOS), liver cirrhosis [2] atherosclerosis, hypertension, and stroke [3]. Moreover, given that IR usually precedes the development of T2DM and contributes to the progressive nature of this challenging and devastating disease, understanding the molecular mechanisms that lead to IR will help facilitate the development of novel therapeutic strategies to prevent or lessen disease progression. However, despite extensive ongoing research into IR, its molecular mechanism(s) of action remains largely elusive.

Recently, research on metabolic processes associated with IR and T2DM has revealed an exciting role for the biochemical and physiological role of zinc and the proteins that transport zinc in cells in diseases associated with abnormal cellular signaling [4]. Accordingly, zinc and the proteins that transport this metal ion have emerged as potential therapeutic targets for disease states associated with dysfunctional metabolism. For example, zinc in the diet and zinc transporter proteins that influence/regulate zinc metabolism are implicated in metabolic homeostasis in peripheral tissues (e.g. skeletal muscle and liver) that respond to insulin [4].

Zinc is ubiquitous in physiological systems, albeit, within tightly controlled parameters, and therefore suggests that atypical levels are likely to have significant biological and

* Correspondence: Stephen.myers@utas.edu.au
Faculty of Health, School of Health Sciences, University of Tasmania, Newnham Campus, Launceston, TAS 7250, Australia

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clinical effects on disease processes. Knowing how zinc transporter proteins and the storage of zinc in cells are involved in metabolic processes implicated in IR for example, may present opportunities to develop novel drugs targeting these transporters to prevent or treat IR and T2DM disease progression.

Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is devastating disorder characterised by hyperinsulinemia, hyperglycaemia, compromised energy metabolism and expenditure, and the progression of chronic illness and disease. T2DM is high complex involving both genetic predisposition and environmental factors. A major factor involved in a person's susceptibility to T2DM can be linked through family history of diabetes. For example, Pacific Islander peoples are a unique population with especially high rates of T2DM [5]. The environment also plays a major role in the development of IR and T2DM with inactivity and poor nutritional status being two key factors [6].

Development of T2DM

The development of T2DM is preceded by IR, a disorder associated with hyperinsulinemia, glucose intolerance and dysfunctional energy metabolism [7]. A leading concern for people with IR is the progressive failure of pancreatic β-cell function (a major determinant of T2DM progression) and thus, compromised insulin secretion [8]. T2DM occurs primarily due to pancreatic β-cell failure, including disruption of β-cell function and mass [8]. Elevated blood glucose causes the pancreatic β-cells to produce more insulin resulting in hyperinsulinemia. Thus, pancreatic β-cells amplify insulin synthesis as well as insulin secretion pathways to overcome IR through an adaptive response called β-cell compensation [9]. Consequently, the failure of β-cells occurs in response to elevated insulin levels and thus elevated blood glucose which results in insulin insufficiency and overt diabetes. Accordingly, T2DM patients with loss of β-cell function will cease to live a normal life and will endure life-long pharmacological intervention, often with episodes of illness from unfavourable side-effects associated with the anti-diabetic treatments. Therefore, prevention strategies that take advantage of this "window of opportunity" (before β-cell failure) to prevent or lessen disease progression would have an enormous impact on the health and wellbeing of our communities.

Current drug treatments for T2DM

There are a range of medicines available to manage and treat IR and T2DM, however, side effects of these drugs have always been a foremost challenge in relation to the goal of pharmacotherapy [10] (Table 1). Yet, treatments for IR have not advanced significantly in the last few decades because of the inadequate knowledge about the pleiotropic effects that these drugs have on specific molecular targets [11]. Therefore, finding strategies to increase the efficacy and safety of therapeutic treatment for IR and T2DM is highly critical. In this context, research over the last decade has suggested a role for zinc in the treatment of T2DM [12]. For example, lowered zinc concentrations have been identified in some patients with T2DM [13] and zinc supplementation appears to improve the effectiveness of oral hypoglycaemic agents, decreasing blood glucose, triglycerides and inflammation in some patients [14]. However, many of these kinds of studies are not consistent in their findings and adds further complications in determining a role for zinc in these processes. Thus, it will be important to look at the role of zinc transporters and how they transport zinc within a cellular context during disease progression.

Table 1 Some common anti-diabetic therapies and their side effects

Current Therapies	Side Effects
Metformin (dimethylbiguanide)	Gastrointestinal intolerance and side effects [76, 77].
Sulphonylureas	Hypoglycaemia risk, weight gain [78], cardiovascular disease [79].
Incretin-based therapies	Arrhythmia [80], pancreatitis [81].
Thiazolidinediones	Risk of heart failure [82].
Dipeptidylpeptidase-4 inhibitors	Heart failure [83].
Sodium-coupled glucose co-transporter (SGLT-2) inhibitors	Dehydration and urinary infections in elderly patients [84].

Zinc

Zinc is an essential trace element that is found in all parts of the body including the fluids and secretions, tissues and organs. Zinc is one of the most abundant trace metals (next to iron) in the human body, containing approximately 2–4 g [15]. The concentration of zinc in tissues is highest in the prostate (approximately 200 μg/ml), followed by the pancreas (approximately 40 μg/ml) and then muscle (approximately 50 μg/ml). In human plasma, there is approximately 14–16 μM of total zinc that is distributed to cells and subcellular organelles [15]. In multicellular organisms, almost all zinc is intracellular with the nucleus harbouring approximately 30–40%, the cytoplasm, organelles and specialised vesicles approximately 50%, and the cell membrane has about 10% [12]. Under normal cellular conditions there is no free zinc and therefore, the compartmentalization and distribution of cellular zinc is highly important and tightly controlled so that zinc homeostasis is maintained with an appropriate cellular concentration and physiological range. This is achieved by a family of zinc transporter proteins and metallothioneins [16, 17].

Metallothioneins and zinc transporters

Two families of zinc transporter proteins and zinc buffering proteins play a critical role in the influx, efflux, buffering and compartmentalization of zinc. These are the zinc transporters (ZnT proteins and Zrt/Irt-like ZIP proteins), and the intracellular zinc-binding metallothionein (MT) proteins [18]. MT proteins are a group of soluble low molecular weight metal binding proteins that buffer and translocate zinc within the cytosol [19]. The ZIP and ZnT[1] zinc transporters belong to a family of transmembrane proteins that control zinc movement and thus zinc concentrations in cells. The ZnT family members (ZnT1–10) are involved in depleting cytosolic zinc by moving this metal ion into intracellular organelles or from the extracellular space while the ZIP family members increase cytosolic zinc by transporting this metal ion from outside the cell or from intracellular organelles (Fig. 1).

ZIP transporter family

The original member of the ZIP family of zinc transporters was identified in *Saccharomyces cerevisiae* (designated ZRT1) based on an amino acid sequence similarity to that of Irt1p, an Fe(II) regulated transporter from *Arabidopsis thaliana* [20, 21]. Consistent with the proposed role of ZRT1 in zinc uptake, zinc uptake was increased when this transporter was overexpressed in yeast cells. Similarly, a mutation that disrupted the function of ZRT1 led to reduced levels of zinc uptake and poor cellular growth in the mutant yeast strain [21].

The ZIP family of zinc transporters have a predicted eight transmembrane domain (TMD) structure and this is orientated with the N- and C-terminal facing the extracytosolic region. Many of these members have a predicted long histidine-rich loop region (HX_n, $n = 3–5$) situated between TMD 3 and TMD 4 that is suggested to serve as a zinc-binding site (Fig. 2) [22, 23]. In mammals, there are at least fourteen ZIP transporters that have critical roles in the transport of zinc into cytoplasm from extracellular sources and intracellular storage compartments such as the Golgi apparatus and endoplasmic reticulum, when the cytosolic zinc is depleted. The ZIP transporters are regulated by intracellular and extracellular zinc concentrations, and hormones and cytokines. They are also expressed in several tissues and cell types and their proteins are localized to specific subcellular compartments and have been extensively reviewed elsewhere [24].

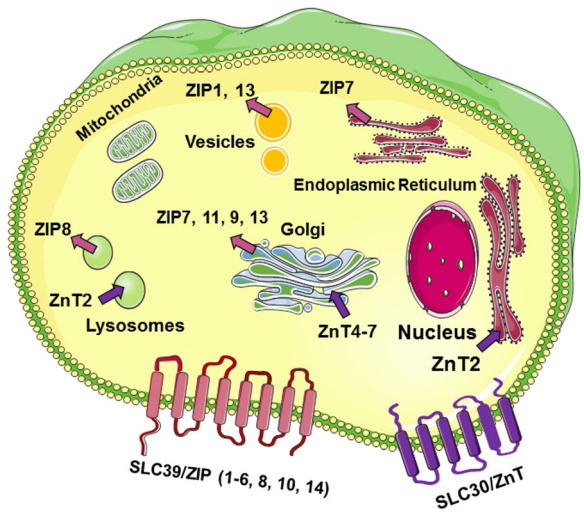

Fig. 1 Cellular and subcellular localization of zinc transporters ZIP and ZnT. The primary localization of ZIP transporters (red arrow heads) and ZnT transporter (purple arrows) is shown representing the direction of zinc transport. Figure was produced using Smart Servier Medical Art, http://smart.servier.com/ and adapted from Kimura and Kambe (2016) [22]

ZnT transporter family

The ZnT zinc transporters are a large family that include members with similar structural homology to bacteria, fungi, nematodes, insects, plants and mammals [25]. This family is predicted to have six transmembrane domains (TMDs) with a histidine-rich loop region between TMD 4 and TMD 5 (Fig. 2). The original mammalian ZnT was identified from a rodent cDNA library and was shown experimentally to confer resistance to zinc toxicity in baby hamster kidney cell lines [26]. Since the discovery of ZnT1, nine other members have been identified (designated SLC30A1–10). ZnTs are implicated in the transport of zinc into subcellular organelles and from the cytosol through the plasma membrane into the extracellular space [25]. Like the ZIPs, the ZnTs are regulated by intracellular and extracellular zinc, hormones, and cytokines and are extensively reviewed elsewhere [24].

Zinc, zinc transporters and cellular signaling: A prelude to cell signaling and insulin resistance

Changes in zinc compartmentalization and availability in cells is typically detected and regulated by the intrinsic control of zinc transporter proteins. The cellular homeostasis of zinc is highly complex and there are several highly significant reviews on these processes [12, 15, 16, 27–30]. Accordingly, this section of the review aims to delineate the mechanisms by which zinc, and zinc transporters contribute to cell signaling and how these processes might provide insights into the molecular mechanisms implicated in disease processes such as insulin resistance and type 2 diabetes.

Zinc mimics the action of several molecules implicated in cellular metabolism including hormones, growth factors, and cytokines, and given the large number of mammalian zinc transporters that regulate zinc homeostasis, it is not surprising that this metal ion has been highlighted as a leading signaling molecule like calcium. Two modes of zinc signaling have been described. These are 1) early

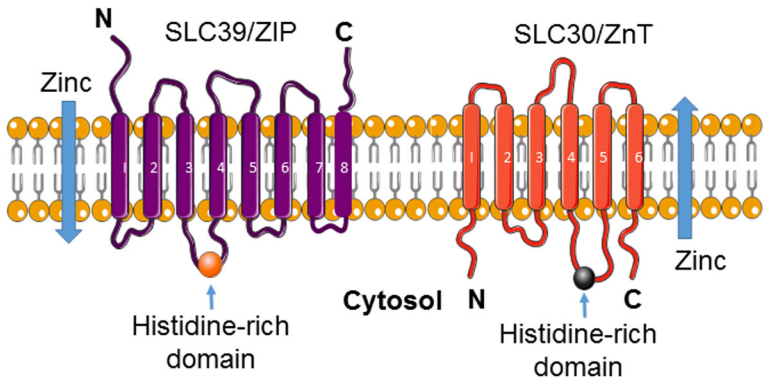

Fig. 2 Predicted structure of the zinc transporters ZIP and ZnT. ZIP transporters are predicted to have eight transmembrane domains (TMDs) with a long histidine loop between TMDs 3 and 4. The ZnT transporters are predicted to have six TMDs with a histidine loop between TMDs 4 and 5. Figure was produced using Servier Medical Art, http://www.servier.com

zinc signaling (EZS) and, 2) late zinc signaling (LZS). EZS, is a process that is independent of gene transcription and results in rapid changes in intracellular levels of zinc that occurs in minutes (the 'zinc wave' response) through the triggered release of zinc from subcellular organelles into the cytosol [31]. This phenomenon was first shown in studies in mast cells where an extracellular stimulation of these cells with the high affinity IgE resulted in a rapid increase in intracellular zinc from the endoplasmic reticulum within minutes [32]. LZS can also be triggered by extracellular stimuli but involves transcriptional-dependent changes in genes and thus proteins that are involved in zinc homeostasis such as storage proteins or transporters [33]. The importance of these two mechanisms of EZS and LZS highlight the diverse roles that this metal ion plays in processes that require rapid signals (such as metabolism) and more long-term functions such as cell differentiation and cell growth.

The defining role of zinc as a signaling molecule was shown in early studies in rat adipocytes where zinc could stimulate lipogenesis, independent, and additive to that of insulin [34]. Similarly, in rat adipocytes, zinc activated cAMP phosphodiesterase and the mobilization of glucose transporters to the plasma membrane, independent of insulin receptor kinase activity [35]. Since these studies implicating zinc as a signaling molecule, there is increasing evidence suggesting zinc acts in extracellular signal recognition [32], second messenger activity [36], protein kinase activity [37], protein phosphorylation [38], and transcription factor regulation [39]. These studies clearly highlight the role of zinc in signaling processes that are also associated with insulin-mediated metabolism.

The mechanisms of the insulin-mimetic action of zinc have been delineated in several studies however it is still unclear how these processes occur. One well-established mechanism of zinc action on cellular signaling events occurs through the inhibition of protein tyrosine phosphatase 1B (PTP1B). PTP1B functions as a negative regulator of insulin and leptin signaling transduction pathways [40]. Thus, the inhibition of PTP1B by zinc ions can prolong the insulin signal through the insulin receptor. Similarly, the ability of zinc to modulate glucose transport, glycogen synthesis, lipogenesis, to inhibit gluconeogenesis and lipolysis, and to regulate key elements of the insulin signaling pathway [41] suggests that this metal ion could provide therapeutic insight or utility in the management and/or treatment of insulin resistance. This is an interesting notion in a clinical context since patients that are insulin resistance have a "blunted" response to insulin and subsequent downstream cellular signaling responses. Therefore, the activation of cellular insulin signaling cascade that is critical to achieve glycemic control might involve zinc. However, the question remains as to whether zinc independently activates critical molecules involved in cellular signaling in the absence of insulin or whether zinc requires insulin for these processes.

Zinc transporters, cellular signaling and insulin resistance

Given the well-established role of zinc transporters in mediating the critical control of zinc homeostasis in cells, it will be important to further delineate their function in cell signaling events in the context of metabolic control. Currently, information is limited to what role the zinc transporters might play in cell signaling events in the context of insulin resistance. Therefore, extrapolation of studies from other cellular systems or disease states that have identified zinc transporters and thus zinc flux in facilitating cell signaling events might prove useful.

Studies accessing the role of zinc transporters in cellular signaling found that the zinc transporters ZnT5 and ZnT7 are responsible for loading zinc to alkaline phosphatases (ALPs) in the biosynthetic-secretory pathway in chicken B

lymphocyte-derived cells [42]. These authors noted that mutant cells lacking both ZnTs resulted in a marked loss in ALP activity and this activity could be restored by over-expressing both ZnT5 and ZnT7. Similarly, the cooperative activity of ZnT1, ZnT4 and metallothioneins are required for the full activation of alkaline ALP in the early-secretory pathway [43]. Accordingly, the above studies demonstrate that ALP can be activated by the ZnT family of zinc transporters and therefore aid in the control of numerous cellular events.

In other studies, ZnT1 can regulate Raf-1 enzymatic activity in *Xenopus* oocytes and cultured mammalian cells [44]. Raf-1 plays a critical role in signal transduction in eukaryote cells where it phosphorylates and activates MEK1, a protein threonine and tyrosine kinase that activates the MAPK family members ERK1 and ERK2 [45]. ZnT1 was shown to bind to the amino end of the Raf-1 protein and promote kinase activation. Moreover, increasing the concentration of intracellular zinc inhibited Ras-mediated signaling through zinc blocking the ability of ZnT1 to bind Raf-1 [44] suggesting that Raf-1 activity requires functional ZnT1.

Of the ZIP transporters, ZIP14 is important in G-protein coupled receptor (GPCR)-mediated signaling through the maintenance of intracellular cAMP levels via suppression of phosphodiesterase activity [46]. In these studies, fasting gluconeogenesis is impaired in the livers of ZIP14 knockout mice which was attributable to changes in GPCR signaling processes. Similarly, in ZIP14 knock-out mouse chondrocytes, parathyroid hormone-related peptide (PTHrP)-mediated *c-fos* activity was significantly reduced. PTHrP stimulates the phosphorylation of cAMP response element-binding protein (CREB) which in turn induces the transcription of *c-fos* [47].

Studies in MCF breast cancer cell lines have identified that ZIP7 is essential in the redistribution of zinc from intracellular stores to the cytoplasm and subsequent zinc-induced inhibition of phosphatases [36]. Moreover, ZIP7 knock-down in these cells prevented the zinc-induced activation of epidermal growth factor receptor (EGFR), insulin-like growth factor-1 receptor (IGF-1R), and protein kinase B (AKT); key molecules implicated in cellular metabolism. In fact, ZIP7 has been coined the "gatekeeper" of cytosolic release from the endoplasmic reticulum (ER) and Golgi apparatus [48]. ZIP7 is phosphorylated by CK2 in MCF-7 cells and this activation leads to the "gated" release of zinc from the ER and the subsequent activation of multiple downstream signaling pathways including AKT and extracellular signal-regulated kinases 1and 2 (ERK1/2) [48].

ZIP7 is essential for the proliferation of intestinal epithelial cells and mice lacking this transporter in intestinal epithelium have massive apoptosis of transit-amplifying cells due to increased endoplasmic reticulum stress (ER) [49]. Similarly, studies identified that the phosphorylation of ZIP7 was increased in cardiomyocytes under hyperglycemia conditions and was implicated in driving ER stress [50]. Given that ZIP7 facilitates the release of zinc from the ER [48] and ablation of ZIP7 in mesenchymal stem cells led to the accumulation of zinc in the ER and subsequent ER dysfunction [51], it is plausible that ZIP7 could also be implicated in ER stress in type 2 diabetes. Undeniably, ER stress and the dysregulation of ER function in pancreatic beta cells are central in the pathogenesis of diabetes [52].

Previously we have identified a role for ZIP7 in glycemic control in skeletal muscle [53]. Skeletal muscle acts as a major reservoir for zinc containing approximately 60% of total whole-body zinc [54]. Knock-down of ZIP7 in C2C12 mouse skeletal muscle cells led to a significant reduction in several genes and proteins involved in glucose metabolism including the insulin receptor (Ir), insulin receptor substrates 1 and 2 (Irs1 and Irs2), the phosphorylation of Akt, glucose transporter Glut4, and glycogen branching enzyme (Gbe) [53]. These data suggest that ZIP7 controls glucose metabolism via the phosphorylation of Akt and Glut4 mobilization (Fig. 3). It is not clear if reduced ZIP7 and thus reduced zinc levels leads to changes associated with the phosphorylation status of the insulin receptor substrates directly, or if reduced ZIP7 leads to inhibition of insulin receptor signaling via binding of PTP-1B. Studies have identified that the ZIPs play a major role in regulating cytosolic zinc homeostasis and insulin secretion [55]. In these studies, it was suggested that the zinc transporters ZIP6 and ZIP7 may have a role in insulin secretion in pancreatic beta cells via alterations in cytosolic and/or subcellular organelle-specific zinc pools. The down regulation of these transporters via knock-down studies led to a significant reduction in glucose-stimulated zinc uptake and oxidative stress in mouse islet cells [55]. These authors speculate that reduced expression of ZIP6 and ZIP7 may disrupt zinc homeostasis and thus produce defects in insulin secretion and beta cell viability that could potentially lead to the development of diabetes.

Recent studies have shown a relationship with ZIP13 and beige adipocyte biogenesis and thermogenesis [56]. In primary white preadipocytes isolated from white fat from ZIP13 null mice, there was a slight increase in common white fat genes but a significant increase in the gene expression of brown-fat specific genes and suggests that this transporter is implicated the inhibition of beige fat differentiation [56]. Moreover, these authors demonstrated that ubiquinated C/EBP-β was decreased in ZIP13 null mice. C/EBP is a major regulator of adipogenesis through the activation of genes essential for mitotic clonal expansion and thus, terminal adipocyte differentiation [57]. Accordingly, these studies suggest that ZIP13 deletion promotes beige adipocyte production and is associated with increased exergy expenditure, reduced diet-induced obesity and insulin resistance.

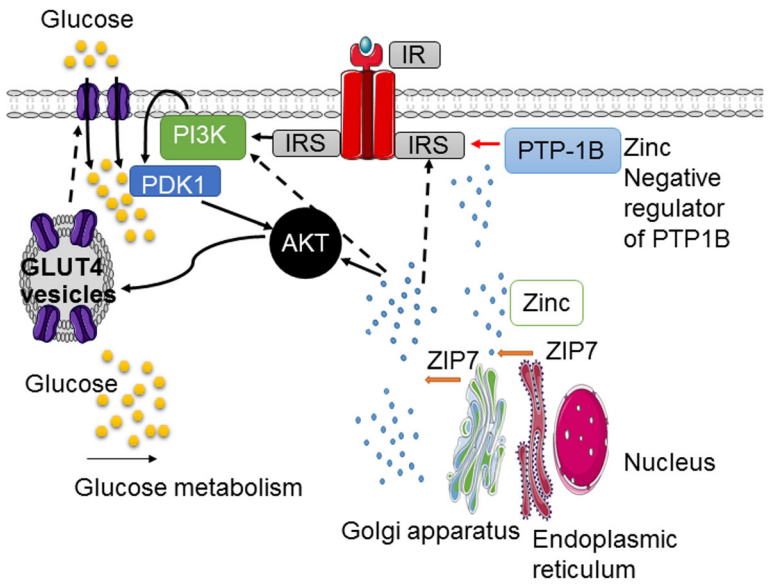

Fig. 3 Potential role of ZIP7-mediated glucose metabolism in skeletal muscle. The ZIP7-gated release of zinc from the Golgi apparatus and/or the endoplasmic reticulum activates the phosphorylation of AKT and the subsequent mobilization of the glucose transporter GLUT4 which in turn brings glucose into the cytosol. Zinc also inactivates the negative regulation of insulin signalling, PTP-1B which allows the insulin signalling cascade process. The role of zinc activation of insulin receptor substrates (IRS) is not known (dashed lines)

ZIP14 has been identified as a critical route for non-transferrin bound iron (NTBI) uptake into liver and pancreatic acinar cells and is essential for the development of liver iron overload in hemochromatosis [58]. Diabetes is frequently associated with hemochromatosis and patients with type 2 diabetes present with high ferritin levels which correlate with diabetic retinopathy [59]. Similarly, ZIP14 knock-out mice have enlarged pancreatic islets, low grade inflammation, and subsequent hyperinsulinemia and increased body fat which are characteristic of type 2 diabetes [60].

Do the zinc transporters and zinc signaling play a role in insulin resistance and the progression of type 2 diabetes?

Zinc plays a major role in many aspects of cell signaling events in several physiological and pathophysiological processes. While it is well established that at least one zinc transporter (ZnT8) is critical for the compartmentalization, structure and secretion of insulin in beta cells of the pancreas [30], there is little information about the other family members in this context. Nonetheless, given the important role that zinc transporters play in delivering bioactive zinc to extracellular, cytosolic and subcellular milieu, and the action of this metal ion on cell signaling events, it is highly tempting to speculate that aberrant storage and release of zinc will result in unfavorable processes associated with insulin signaling and glycemic control.

ZnT8 and type 2 diabetes

Zinc is critical for the physiological role of insulin in the form of storage in the secretory granules of the pancreas as an inactive zinc-insulin hexamer [61, 62]. When the zinc-insulin hexamer is released into the blood circulation, a change in pH drives the dissociation of the complex into a bioactive monomer of insulin [63]. The zinc transporter that initializes zinc movement into insulin granules of the pancreatic β-cells is ZnT8. In fact, this transporter is almost exclusively localized in pancreatic β-cells and it is critical for the synthesis, storage and action of insulin [64].

Genome-wide association studies (GWAS) have discovered that a nonsynonymous single nucleotide polymorphism (SNP) in ZnT8 (rs13266634) encodes a C → T base substitution resulting in a change in the coded protein (p.Arg325Trp) and the production of two protein variants R and W of which the C allele (R variant) is associated with susceptibility to type 2 diabetes [65]. The frequency of the diabetes risk R allele is 91.5%, 71.7% and 56.7% in Africans, Europeans, and Asians, respectively [66]. Moreover, the corresponding at-risk R325 variant had reduced zinc transporter activity compared to the W325 ZnT8 in pancreatic beta-cell lines. Therefore, carriers of the R325 variant may have compromised packaging of insulin in pancreatic beta-cell granules [67]. In studies of 846 European individuals, each of whom had a parent with type 2 diabetes, it was demonstrated that homozygous carriers of the major C risk-allele variant had compromised pancreatic β-cell insulin secretion following an intravenous glucose load [68]. It was suggested by these authors that the function and/or

production of ZnT8 in carriers of the C risk allele is reduced and therefore likely to contribute to compromised pancreatic beta-cell function.

Although GWAS studies have been successful in identifying variant ZnT8 alleles, it does not necessarily imply that the risk allele has a direct pathophysiological effect on beta-cell insulin secretion. A Meta-Analysis of Glucose and Insulin-related traits Consortium (MAGIC) was recently formed to conduct large-scale meta-analyses of genome-wide data for continuous diabetes-related traits in non-diabetics [69]. Meta-analyses were performed on approximately 2.5 M directly genotyped or imputed SNPs from twenty-one GWAS that were informative for fasting glucose (FG), fasting insulin (FI) and, pancreatic beta-cell function (HOMA-B) and insulin resistance (HOMA-IR) in non-diabetic participants [69]. It was identified that the risk allele for ZnT8 was associated with higher FG levels and an increase in two-hour glucose response in non-diabetics. Although these studies identified several genetic glycemic risk loci for type 2 diabetes, including ZnT8, not all loci are associated with pathological levels of glucose and type 2 diabetes risk.

More recent studies that sequenced approximately 150,000 individuals across five ancestry groups reported twelve rare protein-truncating mutations in ZnT8 which together explain a 65% reduced risk of developing type 2 diabetes [70]. It was found that a nonsense variant encoding (c.412C → T, p.Arg138*) heterozygosity yielded a 53% reduction in type 2 diabetes risk. Similarly, heterozygosity for the variant encoding p.Lys34Serfs*50 which is predicted to cause a frameshift and loss of all six ZnT8 transmembrane domains was associated with an 80% reduction in type 2 diabetes risk.

Recently, studies aimed to delineate the effect of dietary factor interactions with ZnT8 polymorphism (rs13266634) and the risk of developing metabolic syndrome found a significant interaction among omega-3 fatty acid consumption and ZnT8 in the context of metabolic syndrome, dyslipidemia, and abdominal obesity [71]. Participants with the CC genotype benefited more from the consumption of omega-3 fatty acids than carriers of the CT + TT genotypes. Carriers of the CC genotype had reduced risk of developing these disease states with increased consumption of omega-3 fatty acids. Moreover, the risk of abdominal obesity in the CT + TT genotype groups increased significantly with salty snack consumption but not in the CC homozygote carriers.

Studies to investigate the role of ZnT8 and glucose homeostasis has been established with ZnT8 null mouse models with global deletion of ZnT8 or pancreatic beta-cell specific ZnT8 deletion [72]. Most models resulted in impaired or unaltered glucose tolerance, however in ZnT8-depleted beta cells insulin granule abnormalities were observed, and this was concomitant with a loss of zinc release from secretory granules [73]. Similarly, ZnT8-specific deletion in beta cells resulted in reduced peripheral insulin concentrations despite an unexpected increase in insulin secretion from isolated ZnT8-depleted islets [74]. These authors suggest that secreted insulin from the pancreas in the ZnT8 knock-out mouse suppresses hepatic insulin clearance and dysregulation of this process could play a role in the pathogenesis of type 2 diabetes. A recent study [75] revealed ZnT8 deletion in mouse beta-cells resulted in a significant impairment in zinc release, normal or increased insulin secretion and subsequent impairment in glucose tolerance. Moreover, transgenic mice that overexpressed ZnT8 in beta cells showed a significant improvement in zinc release, lower levels of insulin secretion and improved glucose tolerance [75].

Given the pancreatic tissue-specificity of ZnT8 there is potential for this transporter to be amendable to therapy in the treatment of diabetes. However, targeting ZnT8 in the treatment of diabetes could prove to be highly complex. Although results from ZnT8 null mice suggest that increasing ZnT8 could improve insulin secretion and glycemic control, loss of function mutations in ZnT8 suggest a protective role for this transporter in type 2 diabetes. While ZnT8 plays a critical role in insulin physiology, and over the last decade or so this transporter has been given much attention for its role in diabetes, other zinc transporters have not had the same focused attention until recently. Apart from the many studies on zinc signaling in cells and the insulin-mimetic action of zinc, it is unclear which zinc transporters are involved in initiating these signaling processes. Clues from studies on the role of ZIP7 as the "gate-keeper" of zinc release from the Golgi apparatus [48] and subsequent ZIP7-mediated cell signaling events in skeletal muscle [49] no doubt place this transporter in an important position for further studies. Identifying how the zinc transporters are implicated in zinc signaling events that are amendable to insulin signaling processes in insulin resistance may help elucidate novel therapeutic options for the treatment of early diabetic symptoms and thus the long-term management of this disorder and associated type 2 diabetes.

Conclusion

Zinc is an essential metal ion that is ubiquitous in many metabolic and physiological processes. The emerging role of zinc as an insulin mimetic in maintaining cellular function suggests that atypical levels, and aberrant compartmentalization, transport and storage of zinc will have biological effects that could be amendable to clinical intervention. Although current understandings on the role of zinc transporters in insulin resistance is not available, and this knowledge is only just emerging in type 2 diabetes, it is clear from studies on ZnT8 that this family of transporters has utility for the development

of novel diabetic therapies. While ZnT8 plays a significant role in insulin biology and therefore represents an attractive target for diabetes therapy, the other members of the zinc transporter family in diabetes are less defined. However, we can speculate from the information presented in this review that the other transporters are involved in processes that facilitate insulin signaling and glycemic control and therefore could offer exciting new targets that are amendable to therapeutic intervention in the treatment of diseases associated with insulin resistance and type 2 diabetes.

Endnotes

[1]For brevity, the terms ZIP and ZnT will be used throughout.

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References

1. DeFronzo RA, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care. 2009;32(Suppl 2):S157–S63.
2. Hojlund K. Metabolism and insulin signaling in common metabolic disorders and inherited insulin resistance. Dan Med J. 2014;61(7):B4890.
3. Taghibiglou C, Rashid-Kolvear F, Van Iderstine SC, Le-Tien H, Fantus IG, Lewis GF, et al. Hepatic very low density lipoprotein-ApoB overproduction is associated with attenuated hepatic insulin signaling and overexpression of protein-tyrosine phosphatase 1B in a fructose-fed hamster model of insulin resistance. J Biol Chem. 2002;277(1):793–803.
4. Myers SA, Nield A, Myers M. Zinc transporters, mechanisms of action and therapeutic utility: implications for type 2 diabetes mellitus. J Nutr Metab. 2012;2012:173712.
5. Zimmet P. Review: epidemiology of diabetes — its history in the last 50 years. Br J Diabetes & Vascular Dis. 2002;2(6):435–9.
6. Kommoju UJ, Reddy BM. Genetic etiology of type 2 diabetes mellitus: a review. Int J Diabetes in Developing Countries. 2011;31(2):51–64.
7. Abdul-Ghani MA, DeFronzo RA. Pathogenesis of Insulin Resistance in Skeletal Muscle. J Biomed Biotechnol 2010;2010 doi:10115.2010.476279.
8. Janikiewicz J, Hanzelka K, Kozinski K, Kolczynska K, Dobrzyn A. Islet beta-cell failure in type 2 diabetes–within the network of toxic lipids. Biochem Biophys Res Commun. 2015;460(3):491–6.
9. Sachdeva MM, Stoffers DA. Minireview: meeting the demand for insulin: molecular mechanisms of adaptive postnatal beta-cell mass expansion. Mol Endocrinol. 2009;23(6):747–58.
10. Kaur N, Kishore L, Singh R. Attenuating diabetes: what really works? Curr Diabetes Rev. 2016;12(3):259–78.
11. Pajvani UB, Accili D. The new biology of diabetes. Diabetologia. 2015;58(11): 2459–68.
12. Miao X, Sun W, Fu Y, Miao L, Cai L. Zinc homeostasis in the metabolic syndrome and diabetes. Front Med. 2013;7(1):31–52.
13. Basaki M, Saeb M, Nazifi S, Shamsaei HA. Zinc, copper, iron, and chromium concentrations in young patients with type 2 diabetes mellitus. Biol Trace Elem Res. 2012;148(2):161–4.
14. Khan MI, Siddique KU, Ashfaq F, Ali W, Reddy HD, Mishra A. Effect of high-dose zinc supplementation with oral hypoglycemic agents on glycemic control and inflammation in type-2 diabetic nephropathy patients. J Nat Sci Biol Med. 2013;4(2):336–40.
15. Jansen J, Karges W, Rink L. Zinc and diabetes — clinical links and molecular mechanisms. J Nutr Biochem. 2009;20(6):399–417.
16. Maret W. Metals on the move: zinc ions in cellular regulation and in the coordination dynamics of zinc proteins. Biometals. 2011;24(3):411–8.
17. Eide DJ. Zinc transporters and the cellular trafficking of zinc. Biochim Biophys Acta. 2006;1763(7):711–22.
18. Myers SA. Zinc transporters and zinc signaling: new insights into their role in type 2 diabetes. Int J Endocrinol. 2015;2015:167503.
19. Guy A, Rutter PC, Bellomo EA, Maret W, Mitchell RK, Hodson DJ, et al. Intracellular zinc in insulin secretion and action: a determinant of diabetes risk? Proc Nutr Soc. 2016;75(01):61–72.
20. Eide D, Broderius M, Fett J, Guerinot MLA. Novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proc Natl Acad Sci. 1996;93(11):5624–8.
21. Zhao H, Eide D. The yeast ZRT1 gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. Proc Natl Acad Sci. 1996;93(6):2454–8.
22. Kimura T, Kambe T. The functions of Metallothionein and ZIP and ZnT transporters: an overview and perspective. Int J Mol Sci. 2016;17(3)
23. Milon B, Wu Q, Zou J, Costello LC, Franklin RB. Histidine residues in the region between transmembrane domains III and IV of hZip1 are required for zinc transport across the plasma membrane in PC-3 cells. Biochim Biophys Acta Biomembr. 2006;1758(10):1696–701.
24. Myers SA, Nield A. Zinc, zinc transporters and type 2 diabetes chapter 2. In: Endocrine disease: iConcept Press Ltd, Hong Kong; 2014. p. 25–48. ISBN:9 78–1–922227.
25. Huang L, Tepaamorndech S. The SLC30 family of zinc transporters – a review of current understanding of their biological and pathophysiological roles. Mol Asp Med. 2013;34(2–3):548–60.
26. Palmiter RD, Findley SD. Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. EMBO J. 1995;14(4):639–49.
27. Fukada T, Yamasaki S, Nishida K, Murakami M, Hirano T. Zinc homeostasis and signaling in health and diseases. J Biol Inorg Chem. 2011;16(7):1123–34.
28. Colvin RA, Holmes WR, Fontaine CP, Maret W. Cytosolic zinc buffering and muffling: their role in intracellular zinc homeostasis. Metallomics. 2010;2(5):306–17.
29. Mocchegiani E, Giacconi R, Malavolta M. Zinc signalling and subcellular distribution: emerging targets in type 2 diabetes. Trends Mol Med. 2008; 14(10):419–28.
30. Kambe T. An overview of a wide range of functions of ZnT and zip zinc transporters in the secretory pathway. Biosci Biotechnol Biochem. 2011; 75(6):1036–43.
31. Bafaro E, Liu Y, Xu Y, Dempski RE. The emerging role of zinc transporters in cellular homeostasis and cancer. Signal Transduct Trageted Ther. 2017;2: e17029. doi:10.1038/sigtrans.2017.29.
32. Yamasaki S, Sakata-Sogawa K, Hasegawa A, Suzuki T, Kabu K, Sato E, et al. Zinc is a novel intracellular second messenger. J Cell Biol. 2007;177(4):637–45.
33. Hirano T, Murakami M, Fukada T, Nishida K, Yamasaki S, Suzuki T. Roles of zinc and zinc signaling in immunity: zinc as an intracellular signaling molecule. In: Frederick WA, editor. Advances in immunology: Academic Press publications, USA; vol 97. 2008. p. 149–76.

34. Coulston L, Dandona P. Insulin-like effect of zinc on adipocytes. Diabetes. 1980;29(8):665–7.
35. Ezaki O. IIb group metal ions (Zn2+, Cd2+, Hg2+) stimulate glucose transport activity by post-insulin receptor kinase mechanism in rat adipocytes. J Biol Chem. 1989;264(27):16118–22.
36. Taylor KM, Vichova P, Jordan N, Hiscox S, Hendley R, Nicholson RI. ZIP7-Mediated Intracellular Zinc Transport Contributes to Aberrant Growth Factor Signaling in Antihormone-Resistant Breast Cancer Cells. Endocrinology. 2008;149(10):4912–20.
37. Tang X-H, Shay NF. Zinc has an insulin-like effect on glucose transport mediated by Phosphoinositol-3-kinase and Akt in 3T3-L1 fibroblasts and adipocytes. J Nutr. 2001;131(5):1414–20.
38. Pandey N, Vardatsikos G, Mehdi M, Srivastava A. Cell-type-specific roles of IGF-1R and EGFR in mediating Zn^{2+}-induced ERK1/2 and PKB phosphorylation. J Biol Inorg Chem. 2010;15(3):399–407.
39. Rutherford JC, Bird AJ. Metal-responsive transcription factors that regulate iron, zinc, and copper homeostasis in eukaryotic cells. Eukaryot Cell. 2004;3(1):1–13.
40. Bellomo E, Massarotti A, Hogstrand C, Maret W. Zinc ions modulate protein tyrosine phosphatase 1B activity. Metallomics. 2014;6(7):1229–39.
41. Vardatsikos G, Pandey NR, Srivastava AK. Insulino-mimetic and anti-diabetic effects of zinc. J Inorg Biochem. 2013;120:8–17.
42. Suzuki T, Ishihara K, Migaki H, Matsuura W, Kohda A, Okumura K, et al. Zinc transporters, ZnT5 and ZnT7, are required for the activation of alkaline phosphatases, zinc-requiring enzymes that are glycosylphosphatidylinositol-anchored to the cytoplasmic membrane. J Biol Chem. 2005;280(1):637–43.
43. Fujimoto S, Itsumura N, Tsuji T, Anan Y, Tsuji N, Ogra Y, et al. Cooperative functions of ZnT1, metallothionein and ZnT4 in the cytoplasm are required for full activation of TNAP in the early secretory pathway. PLoS One. 2013;8(10):e77445.
44. Jirakulaporn T, Muslin AJ. Cation diffusion facilitator proteins modulate Raf-1 activity. J Biol Chem. 2004;279(26):27807–15.
45. Brietz A, Schuch KV, Wangorsch G, Lorenz K, Dandekar T, Analyzing ERK. 1/2 signalling and targets. Mol BioSyst. 2016;12:2436.
46. Hojyo S, Fukada T, Shimoda S, Ohashi W, Bin B-H, Koseki H, et al. The zinc transporter SLC39A14/ZIP14 controls G-protein coupled receptor-mediated signaling required for systemic growth. PLoS One. 2011;6(3):e18059.
47. Guo J, Iida-Klein A, Huang X, Abou-Samra AB, Segre GV, Bringhurst FR. Parathyroid hormone (PTH)/PTH-related peptide receptor density modulates activation of phospholipase C and phosphate transport by PTH in LLC-PK1 cells. Endocrinology. 1995;136(9):3884–91.
48. Taylor KM, Hiscox S, Nicholson RI, Hogstrand C, Kille P. Protein kinase CK2 triggers cytosolic zinc signaling pathways by phosphorylation of zinc channel ZIP7. Sci Signal. 2012;5(210):ra11.
49. Ohashi W, Kimura S, Iwanaga T, Furusawa Y, Irie T, Izumi H, et al. Zinc transporter SLC39A7/ZIP7 promotes intestinal epithelial self-renewal by resolving ER stress. PLoS Genet. 2016;12(10):e1006349.
50. Tuncay E, Bitirim V, Durak A, Carrat G, Taylor K, Rutter G, et al. Hyperglycemia-induced changes in ZIP7 and ZnT7 expression cause Zn2+ release from the Sarco(endo)plasmic reticulum and mediate ER-stress in the heart. Diabetes. 2017;66(5):1346–58.
51. Bum-Ho B, Jinhyuk B, Juyeon S, Se-Young K, Eunyoung L, Kyuhee P, et al. Requirement of zinc transporter SLC39A7/ZIP7 for dermal development to fine-tune endoplasmic reticulum function by regulating protein disulfide isomerase. J Investig Dermatol. 2017;137(8):1682–91.
52. Cnop M, Toivonen S, Igoillo-Esteve M, Salpea P. Endoplasmic reticulum stress and eIF2alpha phosphorylation: the Achilles heel of pancreatic beta cells. Mol Metab. 2017;6(9):1024–39.
53. Myers SA, Nield A, Chew GS, Myers MA. The zinc transporter, Slc39a7 (Zip7) is implicated in glycaemic control in skeletal muscle cells. PLoS One. 2013;8(11):e79316.
54. Hara T, Takeda T, Takagishi T, Fukue K, Kambe T, Fukada T. Physiological roles of zinc transporters: molecular and genetic importance in zinc homeostasis. J Physiol Sci. 2017;67(2):283–301.
55. Liu Y, Batchuluun B, Ho L, Zhu D, Prentice KJ, Bhattacharjee A, et al. Characterization of zinc influx transporters (ZIPs) in pancreatic beta cells: roles in regulating cytosolic zinc homeostasis and insulin secretion. J Biol Chem. 2015;290(30):18757–69.
56. Fukunaka A, Fukada T, Bhin J, Suzuki L, Tsuzuki T, Takamine Y, et al. Zinc transporter ZIP13 suppresses beige adipocyte biogenesis and energy expenditure by regulating C/EBP-β expression. PLoS Genet. 2017;13(8):e1006950.
57. Liu Y, Zhang YD, Guo L, Huang HY, Zhu H, Haung JX, et al. Protein inhibitor of activated STAT 1 (PIAS1) is identified as the SUMO E3 ligase of CCAAT/enhancer-binding protein beta (C/EBPbeta) during adipogenesis. Mol Cell Biol. 2013;33(22):4606–17.
58. Jenkitkasemwong S, Wang CY, Coffey R, Zhang W, Chan A, Biel T, et al. SLC39A14 is required for the development of hepatocellular iron overload in murine models of hereditary hemochromatosis. Cell Metab. 2015;22(1):138–50.
59. Altamura S, Kopf S, Schmidt J, Mudder K, da Sila AR, Nawroth P, et al. Uncoupled iron homeostasis in type 2 diabetes mellitus. J Mol Med. 2017; doi10.1007/s00109-017-1596-3.
60. Aydemir TB, Troche C, Kim MH, Cousins RJ. Hepatic ZIP14-mediated Zinc Transport Contributes to Endosomal Insulin Receptor Trafficking and Glucose Metabolism. J Biol Chem. 2016;291(46):23939–51.
61. Chimienti F, Favier A, Seve M. ZnT-8, a pancreatic Beta-cell-specific zinc transporter. Biometals. 2005;18(4):313–7.
62. Wijesekara N, Chimienti F, Wheeler MB. Zinc, a regulator of islet function and glucose homeostasis. Diabetes Obes Metab. 2009;11:202–14.
63. Xu Y, Yan Y, Seeman D, Sun L, Dubin PL. Multimerization and aggregation of native-state insulin: effect of zinc. Langmuir. 2012;28(1):579–86.
64. Wijesekara N, Dai FF, Hardy AB, Giglou PR, Bhattacharjee A, Koshkin V, et al. Beta cell-specific Znt8 deletion in mice causes marked defects in insulin processing, crystallisation and secretion. Diabetologia. 2010;53(8):1656–68.
65. Sladek R, Rocheleau G, Rung J, Dina C, Shen L, Serre D, et al. A genome-wide association study identifies novel risk loci for type 2 diabetes. Nature. 2007;445(7130):881–5.
66. Maruthur NM, Mitchell BD. Zinc-rs13266634 and the arrival of diabetes pharmacogenetics: the "zinc mystique". Diabetes. 2014;63(5):1463–4.
67. Nicolson TJ, Bellomo EA, Wijesekara N, Loder MK, Baldwin JM, Gyulkhananyan AV, et al. Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes–associated variants. Diabetes. 2009;58(9):2070–83.
68. Boesgaard TW, Žilinskaitė J, Vänttinen M, Laakso M, Jansson P-A, Hammarstedt A, et al. The common SLC30A8 Arg325Trp variant is associated with reduced first-phase insulin release in 846 non-diabetic offspring of type 2 diabetes patients—the EUGENE2 study. Diabetologia. 2008;51(5):816–20.
69. Dupuis J, Langenberg C, Prokopenko L, Saxena R, Soranzo N, Jackson AU, et al. New genetic loci implicated in fasting glucose homeostasis and their impact on type 2 diabetes risk. Nat Genet. 2010;42(2):105–16.
70. Flannick J, Thorleifsson G, Beer NL, Jacobs SB, Grarup N, Burtt NP, et al. Loss-of-function mutations in SLC30A8 protect against type 2 diabetes. Nat Genet. 2014;46(4):357–63.
71. Hosseini-Esfahani F, Mirmiran P, Koochakpoor G, Daneshpour MS, Guity K, Azizi F. Some dietary factors can modulate the effect of the zinc transporters 8 polymorphism on the risk of metabolic syndrome. Sci Rep. 2017;7(1):1649.
72. Chabosseau P, Rutter GA. Zinc and diabetes. Arch Biochem Biophys. 2016; 611(Supplement C):79–85.
73. Lemaire K, Ravier MA, Schraenen A, Creemers JWM, Van de Plas CR, Granvik M, et al. Insulin crystallization depends on zinc transporter ZnT8 expression, but is not required for normal glucose homeostasis in mice. PNAS. 2009; 106(35):14872–7.
74. Tamaki M, Fujitani Y, Hara A, Uchida T, Tamura Y, Takeno K, et al. The diabetes-susceptible gene SLC30A8/ZnT8 regulates hepatic insulin clearance. J Clin Invest. 2013;123(10):4513–24.
75. Mitchell RK, Hu M, Chabosseau PL, Cane MC, Meur G, Bellomo EA, et al. Molecular genetic regulation of Slc30a8/ZnT8 reveals a positive association with glucose tolerance. Mol Endocrinol. 2016;30(1):77–91.
76. McCreight LJ, Bailey CJ, Pearson ER. Metformin and the gastrointestinal tract. Diabetologia. 2016;59(3):426–35.
77. Dujic T, Zhou K, Donnelly LA, Tavendale R, Palmer CN, Pearson ER. Association of Organic Cation Transporter 1 with intolerance to metformin in type 2 diabetes: a GoDARTS study. Diabetes. 2015;64(5):1786–93.
78. Lim PC, Chong CP. What's next after metformin? Focus on sulphonylurea: add-on or combination therapy. Pharm Pract. 2015;13(3):606.
79. Phung OJ, Schwartzman E, Allen RW, Engel SS, Rajpathak SN. Sulphonylureas and risk of cardiovascular disease: systematic review and meta-analysis. Diabet Med. 2013;30(10):1160–71.
80. Wang T, Wang F, Zhou J, Tang H, Giovenale S. Adverse effects of incretin-based therapies on major cardiovascular and arrhythmia events: meta-analysis of randomized trials. Diabetes Metab Res Rev. 2016;32(8):843–57.

81. Li L, Shen J, Bala MM, Busse JW, Ebrahim S, Vandvik PO, et al. Incretin treatment and risk of pancreatitis in patients with type 2 diabetes mellitus: systematic review and meta-analysis of randomised and non-randomised studies. BMJ. 2014;348:g2366.
82. Gilbert RE, Krum H. Heart failure in diabetes: effects of anti-hyperglycaemic drug therapy. Lancet. 2015;385(9982):2107–17.
83. Scirica BM, Bhatt DL, Braunwald E, Steg PG, Davidson J, Hirshberg B, et al. Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus. N Engl J Med. 2013;369(14):1317–26.
84. Haas B, Eckstein N, Pfeifer V, Mayer P, Hass MDS. Efficacy, safety and regulatory status of SGLT2 inhibitors: focus on canagliflozin. Nutr Diabetes. 2014;4(11):e143.

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CHAPTER 3

ZINC STIMULATES GLUCOSE OXIDATION AND GLYCEMIC CONTROL BY MODULATING THE INSULIN SIGNALLING PATHWAY IN HUMAN AND MOUSE SKELETAL MUSCLE CELLS

PLOS ONE

RESEARCH ARTICLE

Zinc stimulates glucose oxidation and glycemic control by modulating the insulin signaling pathway in human and mouse skeletal muscle cell lines

Shaghayegh Norouzi, John Adulcikas, Sukhwinder Singh Sohal, Stephen Myers*

College of Health and Medicine, School of Health Sciences, University of Tasmania, Newnham Campus, Launceston, Tasmania, Australia

* stephen.myers@utas.edu.au

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Abstract

Zinc is a metal ion that is an essential cell signaling molecule. Highlighting this, zinc is an insulin mimetic, activating cellular pathways that regulate cellular homeostasis and physiological responses. Previous studies have linked dysfunctional zinc signaling with several disease states including cancer, obesity, cardiovascular disease and type 2 diabetes. The present study evaluated the insulin-like effects of zinc on cell signaling molecules including tyrosine, PRSA40, Akt, ERK1/2, SHP-2, GSK-3β and p38, and glucose oxidation in human and mouse skeletal muscle cells. Insulin and zinc independently led to the phosphorylation of these proteins over a 60-minute time course in both mouse and human skeletal muscle cells. Similarly, utilizing a protein array we identified that zinc could active the phosphorylation of p38, ERK1/2 and GSK-3B in human and ERK1/2 and GSK-3B in mouse skeletal muscle cells. Glucose oxidation assays were performed on skeletal muscle cells treated with insulin, zinc, or a combination of both and resulted in a significant induction of glucose consumption in mouse ($p<0.01$) and human ($p<0.05$) skeletal muscle cells when treated with zinc alone. Insulin, as expected, increased glucose oxidation in mouse ($p<0.001$) and human (0.001) skeletal muscle cells, however the combination of zinc and insulin did not augment glucose consumption in these cells. Zinc acts as an insulin mimetic, activating key molecules implicated in cell signaling to maintain glucose homeostasis in mouse and human skeletal muscle cells. Zinc is an important metal ion implicated in several biological processes. The role of zinc as an insulin memetic in activating key signaling molecules involved in glucose homeostasis could provide opportunities to utilize this ion therapeutically in treating disorders associated with dysfunctional zinc signaling.

Introduction

Insulin resistance is a common pathophysiological condition in which patients present with perturbed biological responses to endogenous insulin leading to compromised glucose homeostasis specifically in liver and skeletal muscle [1]. The contribution of insulin resistance in various diseases such as type 2 diabetes (T2D), obesity, liver cirrhosis, atherosclerosis and cardiovascular

disease [1, 2] is highly significant. A foremost concern for people with insulin resistance is the progressive failure of pancreatic β-cell function (a major determinant of type 2 diabetes progression) and compromised insulin secretion. Therefore, prevention strategies that take advantage of this "window of opportunity" (before β-cell failure) to prevent or lessen disease progression would have an enormous impact on the health and wellbeing of our communities. Currently, zinc is being investigated for its role in cell signaling pathways that are amendable to glucose homeostasis and thus have implications for insulin resistance and type 2 diabetes [3].

Zinc is present in all parts of the body including organs, tissues, fluids and secretions [4] and plays a critical role in a wide variety of biological processes [5, 6]. For example, zinc has a unique and extensive role in nucleic acid and lipid metabolism, cell signaling, growth and differentiation, apoptosis, enzyme activity, and brain and immune function [7]. Normal zinc homeostasis has a critical role in the release and action of insulin to maintain glucose homeostasis [8] since zinc has insulin mimetic activity and controls cellular processes including insulin receptor signal transduction, and insulin storage and secretion [9].

Zinc is essential for the processing, crystallization, and storage of insulin in pancreatic β-cells through the function of the pancreatic zinc transporter ZnT8 that moves zinc into insulin secretory cells [10, 11]. Beta-cell specific ZnT8 knock out mice display glucose intolerance, abnormal β-cell morphology, reduced islet insulin processing, a reduction in the total number of granules, and an increase in empty atypical granules suggesting that insulin crystallization and packaging is compromised [10]. In fact, there is a strong association between a mutation in ZnT8 (an arginine is replaced with a tryptophan at position 325 [R325W] in the cytoplasmic domain) which increases the risk of type 2 diabetes [12,13].

Early studies on zinc's insulin mimetic activity revealed that this metal ion increased glucose metabolism in isolated rat adipocytes [14]. Similarly, in 3T3-L1 adipocyte cells, zinc treatment increased tyrosine phosphorylation of the insulin receptor β subunit and phosphorylation of Akt, and this was concomitant with enhanced glucose transport, independent of insulin [15]. These studies were further supported in C2C12 skeletal muscle cells where zinc could phosphorylate tyrosine, and the insulin receptor substrate 1 (ISR1) in the absence of insulin [16]. Moreover, zinc stimulated glucose consumption in normal and insulin-resistant L6 myotubes and was concomitant with the upregulation of Akt, the translocation of Glut4 and the phosphorylation of Gsk3β [17]. In delineating the mechanism whereby zinc exerts its effect on tyrosine phosphorylation, it was shown that zinc can increase tyrosine phosphorylation by inhibiting tyrosine phosphatases in C6 glioma cells [18,19]. These data are supported by recent studies that showed zinc ions inhibited PTP1B activity through direct binding to the enzyme [20]. These above studies on zinc as an insulin mimetic relay important information into the complexities of the role of this metal ion in signaling pathways and provide justification to further explore these pathways in more detail in metabolic processes associated with glycemic control.

Given the role of zinc as an insulin mimetic in controlling cellular metabolism, we sought to delineate the role of this metal ion in cellular processes amendable to controlling glucose homeostasis in skeletal muscle cells. We identified that in mouse and human skeletal muscle cells, zinc could affect several insulin-dependent cell signaling pathways that was concomitant with increased glucose oxidation.

Materials and methods

Antibodies

Akt (#9272), phospho-Akt (Ser473; #4058), phosphor-p44/42 MAPK (Erk1/2, Thr201/Tyr204; #8544), phospho-p44/42 MAPK (Eek1/2; #9102), SHP-2 (#3752), phospho-SHP-2 (Tyr580;

#3703), phospho-Tyrosine (P-Tyr-100; #5465), GAPDH (#2118), and HRP-linked secondary antibodies (Anti-rabbit #7074; Anti-mouse #7067) were obtained from Cell Signaling Technology, USA. Intracellular signaling array kit (PathScan #7323) was also purchased from Cell Signaling Technology.

Cell culture

Mouse C2C12 cells (a generous gift from Professor Steve Rattigan, Menzies Institute for Medical Research, Hobart, Australia) and human skeletal muscle cells (Thermo Fisher, Australia; catalog number A12555) were cultured in DMEM (Thermo Fisher) medium containing 10% fetal calf serum (FCS) and 100 U/mL penicillin/streptomycin (Thermo Fisher) and were maintained at 37˚C and 5% CO_2 in a humidified atmosphere. Cells were treated with insulin (10 nM), zinc (20 μM) and/or zinc pyrithione (10 μM) (Sigma Aldrich, Australia) for 60 minutes. The dose of zinc used is consistent with previous studies [21–23, 3] and represents the physiological level of plasma zinc which can range from 10.7 μM following a morning fast to 21.1 μM in healthy individuals [24].

Seventy-two hours before treatment, skeletal muscle cells were differentiated into myotubes by the addition of media containing 2% horse serum (Thermo Fisher). Three hours before treatments, the cells were exposed to serum-free conditions.

Immunoblot analysis

Whole cell lysates were prepared in RIPA Lysis buffer in the presence of protease and protein phosphatase inhibitors (Thermo Fisher). Lysates were vortexed every 10 minutes for 1 hour at 4˚C, and centrifuging at 15000 rpm for 5 minutes. Protein concentrations of the supernatants were determined using the BCA assay kit as per manufacturer's instructions (Thermo Fisher). For western blotting analysis, equal amounts of proteins were heated to 95˚C, separated on 4–15% SDS-polyacrylamide gels (Bio-Rad, Australia) and wet transferred to PVDF at 200 mV for 1 hour at 4˚C (Polyvinylidene Difluoride) membranes (Thermo Fisher). The membrane was then blocked 2 hours in TBST (50 mmol/L Tris-Cl, pH 7.6, 150 mmol/L NaCl and 0.1% Tween 20) containing 5% (w/v) casein, and then incubated with the appropriate primary antibody as indicated overnight at 4˚C. The membrane was washed four times with TBST, then incubated with HRP-conjugated secondary antibody for 1 hour. The membrane was again washed four times with TBST, and the blots were developed using SuperSignal West Femto kit [25] (Thermo Fisher). All phosho-immunoreactive species, Akt, ERK, SHP were normalized against total Akt, ERK or SHP. Immunoreactive phospho-tyrosine was normalized to GAPDH.

Intracellular signaling array

The intracellular signaling protein array kit (chemiluminescent Readout) is a slide-based antibody array founded upon the sandwich immunoassay principle. The array kit allows for the simultaneous detection of 18 important signaling molecules when phosphorylated or cleaved. Skeletal muscle cells were treated with 10 nM insulin, 20 μM $ZnSO_4$ in the presence of 10 μM sodium pyrithione (NaPy), 20 μM $ZnSO_4$ alone, or no treatment (as a control) over 30 minutes and cells were lysed in RIPA lysis buffer supplemented with protease and protein phosphatase inhibitors. The assay procedure was performed according to the manufacturer's protocol. Briefly, cell lysates were incubated on the array slide followed by a biotinylated detection antibody cocktail. Streptavidin-conjugated HRP and LumiGLO reagent were then used to visualize the bound detection antibody by chemiluminescence. An image of the slide was captured with a digital imaging system. Spot intensities for each phosho-immunoreactive protein in the

insulin and zinc treated extracts were normalized to their untreated control. The image was analyzed, and the spot intensities quantified using densitometry array analysis software, Image J (https://imagej.nih.gov/ij/).

Glucose oxidation assay

To determine the amount of glucose consumption by cells, glucose oxidase (GOx) activity assay kit was used as per manufacturer's instructions (Sigma). To establish the glucose oxidation assay, cells were cultured in 96 well plate to reach 100% confluence. As the assay medium should be without any exogenous fuel substrate supplementation, cells were cultured in the serum-free media for another 24 hours. After 24 hours serum-free condition, cells were treated with 10 nM insulin, 20 μM of $ZnSO_4$ in the presence of 10 μM NaPy, a combination of 10 nM insulin and 20 μM $ZnSO_4$ in the presence of 10 μM pyrithione, and DMEM alone as a control. The concentration of glucose added to initiate glucose oxidation was 10 mM, which was determined in preliminary experiments to be above saturation. After incubation for 3 hours, cell lysate was collected and reaction mixture (GOx assay buffer, GOx developer, fluorescent peroxidase substrate and GOx substrate) was added and the absorbance was measured at 570 nm using a TECAN infinite M200 PRO flow cytometer. The background was corrected subtracting the blank measurement value from the sample measurement value.

Insulin receptor inhibition

C2C12 skeletal muscle cells were treated with an insulin receptor tyrosine kinase inhibitor HNMPA-$(AM)_3$ (abcam, Australia) at 0, 25, 50 and 100 μM for 1 hour followed by treatment of cells with either insulin (100 nM) for 30 mins, or zinc (20 μM) plus NaPy (10 μM) for 30 minutes. Following treatment, whole cell lysates were prepared and immunoblot analysis was performed on pAkt and total Akt as previously described in the methods above.

Statistical analysis

Data, represented as the means ± SEM, were analyzed by the one-way ANOVA for multiple comparisons using the Graph Pad Prism 5 software to determine any significant differences. $P < 0.05$ was considered significant.

Results

Effect of zinc on insulin-dependent cell signaling molecules Akt, Tyrosine, SHP, ERK1/2, and PRAS40

We measured the phosphorylation status of several proteins implicated in the insulin-signaling cascade in the presence of insulin or zinc. To assess that skeletal muscle cells responded to insulin we measured the phosphorylation status of protein kinase B (Akt), a well-established protein that is phosphorylated in the presence of insulin [26]. Skeletal muscle cells were treated with 10 nM insulin over 60 minutes and subsequent western blots were performed for the immunoreactivity of p-Akt. We observed that 10 nM of insulin-activated p-Akt in both C2C12 mouse and human skeletal muscle cells and thus confirmed our cells responded to insulin treatment (Fig 1). To test whether zinc has insulin mimetic activity to induce the phosphorylation of Akt, skeletal muscle cells were also treated with 20 μM $ZnSO_4$ in the presence of 10 μM pyrithione. Similarly, zinc induced the phosphorylation of Akt within 15 minutes of treatment in both mouse and human cell lines (Fig 1).

The components of the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) pathway are modifiers of cellular insulin responsiveness [27], and

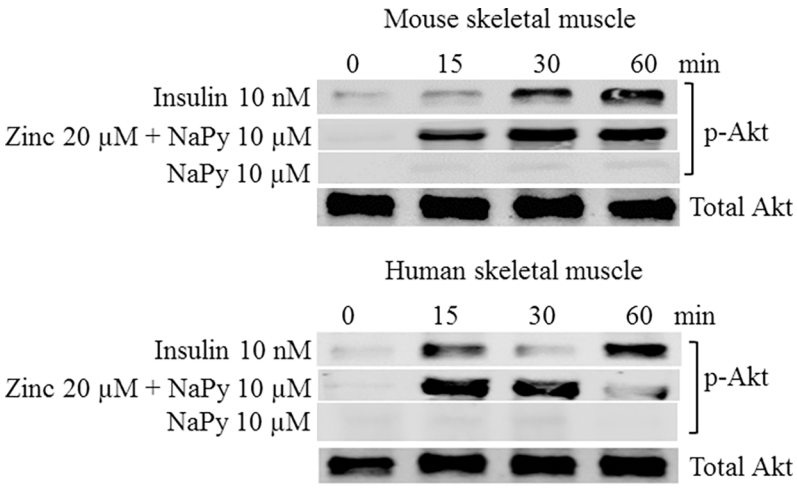

Fig 1. Analysis of p-Akt in mouse and human skeletal muscle cells treated with insulin, zinc, and NaPy over 60 min. Top and bottom panel represents mouse and human skeletal muscle cells, respectively. Time is shown in minutes from 0, 15, 30 and 60 and total Akt was used as an internal loading control in both panels and levels of pAKT were normalized to total Akt.

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therefore sensitive to insulin. Accordingly, we compared the phosphorylation status of ERK1/2 in cells treated with insulin and $ZnSO_4$. Insulin induced the phosphorylation of ERK1/2 within 15 minutes of treatment in mouse and human skeletal muscle cells (Fig 2). Similarly, $ZnSO_4$ in the presence of pyrithione induced ERK phosphorylation within 15 minutes of treatment in these cell lines (Fig 2).

We also identified that treatment of mouse and human skeletal muscle cells with insulin and zinc led to the phosphorylation of SHP-2, a protein tyrosine phosphatase involved in insulin signaling pathway [28] (Fig 3). Insulin and $ZnSO_4$ induced pSHP-2 within 30 minutes of treatment in mouse and human skeletal muscle cells. pSHP-2 increased at 60 minutes of treatment in the human skeletal muscle cells, but not in mouse skeletal muscle (Fig 3).

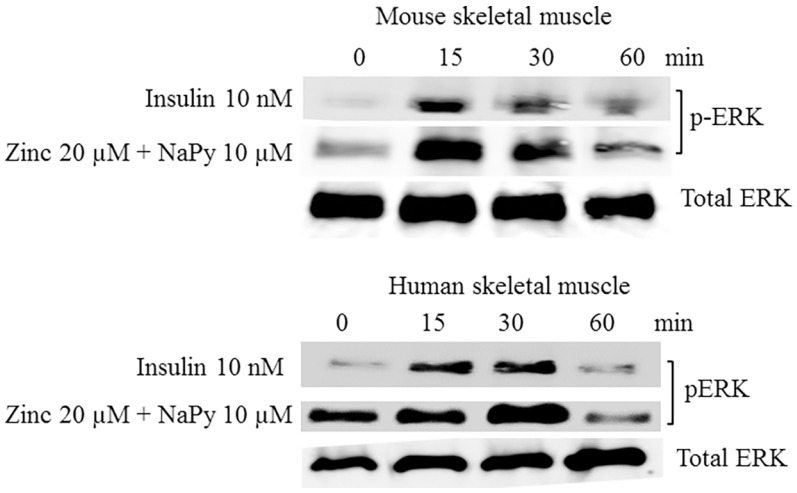

Fig 2. Analysis of p-ERK in mouse and human skeletal muscle cells treated with insulin, zinc, and NaPy over 60 min. Top and bottom panel represents mouse and human skeletal muscle cells, respectively. Time is shown in minutes from 0, 15, 30 and 60 and total ERK was used as an internal loading control in both panels and levels of p-ERK were normalized to total ERK.

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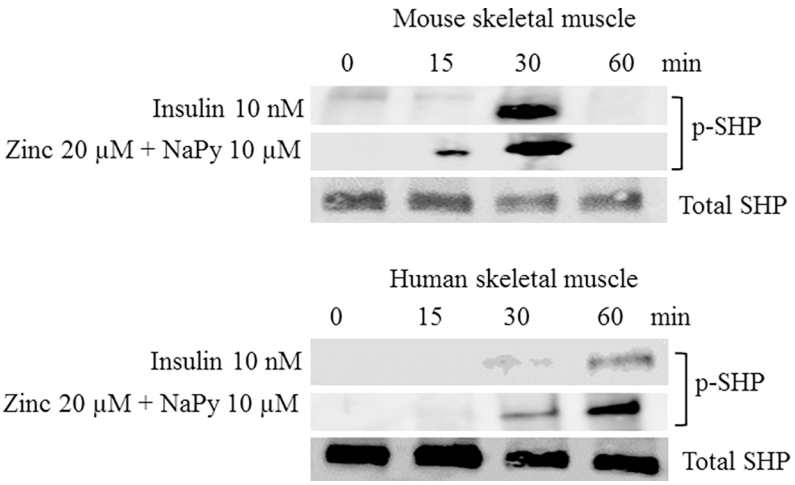

Fig 3. Analysis of p-SHP in mouse and human skeletal muscle cells treated with insulin, zinc, and NaPy over 60 min. Top and bottom panel represents mouse and human skeletal muscle cells, respectively. Time is shown in minutes from 0, 15, 30 and 60 and total SHP was used as an internal loading control in both panels and levels of p-SHP were normalized to total SHP.

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The activation and/or transduction of many signaling pathways including insulin signaling depends on the phosphorylation of tyrosine [29]. To test whether zinc plays a role in the phosphorylation of tyrosine residues in skeletal muscle, we treated mouse and human cells with insulin or $ZnSO_4$ over 60 minutes. Insulin and $ZnSO_4$ induced the phosphorylation of tyrosine residues over the time course in both mouse and human skeletal muscle cell lines (Fig 4).

We also detected significant changes in the phosphorylation of Akt, ERK1/2, glycogen synthesis kinase-3 (GSK-3β), Protein-Rich Akt Substrate of 40 kDa (PRAS40), and p38 using an Intracellular signaling protein array (Table 1 and Fig 5A and 5B). Positive signals for PRSA40, ERK1/2, Akt and GSK-3β were detected in skeletal muscle cells in the mouse protein array when compared to the control panel. Insulin activated PRAS40 and Akt, while $ZnSO_4$ activated PRAS40, ERK1/2, Akt and GSK-3β in this system (Fig 5A). In the human protein array, we observed that insulin activated ERK1/2, Akt and PRAS40 when compared to the control. $ZnSO_4$ activated ERK1/2, Akt, GSK-3β and p38 but not PRAS40 in this instance (Fig 5B).

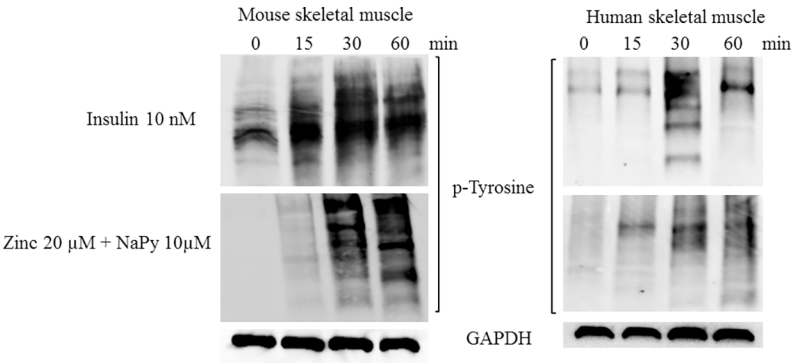

Fig 4. Analysis of p-tyrosine in mouse and human skeletal muscle cells treated with insulin, $ZnSO_4$, and NaPy over 60 min. Top and bottom panel represents mouse and human skeletal muscle cells, respectively. Time is shown in minutes from 0, 15, 30 and 60 and GAPDH was used as an internal loading control in both panels and levels of p-tyrosine were normalized to GAPDH.

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Table 1. Protein target map of intracellular signaling array (cell signaling).

Positive control	ERK1/2	ERK1/2	Stat1	Stat1	Positive control
Stat3	Stat3	Akt (Thr308)	Akt (Thr308)	Akt (Ser473)	Akt (Ser473)
AMPKα	AMPKα	S6 Ribosomal Protein	S6 Ribosomal Protein	mTOR	mTOR
HSP27	HSP27	Bad	Bad	p70 S6 Kinase	p70 S6 Kinase
PRAS40	PRAS40	p53	p53	p38	p38
SAPK/JNK	SAPK/JNK	PARP	PARP	Caspase-3	Caspase-3
Positive control	GSK-3β	GSK-3β	Negative control	Negative control	Negative control

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Densitometry was performed on the protein arrays to determine the level of protein expression between the insulin and $ZnSO_4$ treatment and the control. In mouse skeletal muscle cell lines, Akt and ERK1/2 protein expression was significantly higher ($p < 0.01$ and $p < 0.001$ respectively) in the $ZnSO_4$ treated cells when compared to the control (Fig 6A and 6B, respectively). GSK-3β expression and activation play a critical role in mammalian cells and impacts various cellular processes including glycogen synthesis and glucose transport [30]. The GSK-3β protein levels were significantly higher ($p < 0.001$) in cells treated with $ZnSO_4$ compared to control (Fig 6C). PRAS40, another important cell signaling molecule, is among the most prominent Akt and mTOR complex 1 (mTORC1) substrates being phosphorylated in response to growth factor stimulation such as insulin in eukaryotes [31]. The PRAS40 protein expression was also significantly higher ($p < 0.001$) in mouse cells treated with $ZnSO_4$ when compared to control (Fig 6D).

In the human cell lines, pAkt expression was significantly different ($p < 0.001$) over the control during $ZnSO_4$ and insulin treatment (Fig 7A). Similarly, pERK1/2 protein expression was significantly different for $ZnSO_4$ treatment ($p < 0.01$) and insulin treatment ($p < 0.001$) over

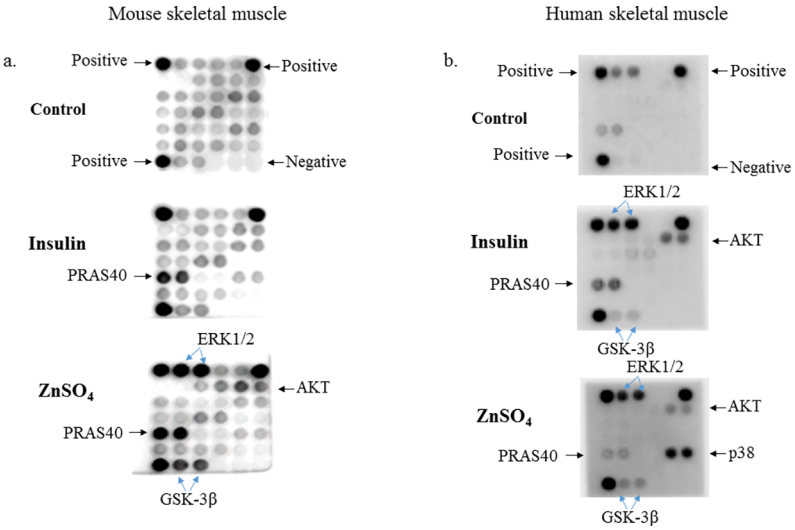

Fig 5. Effect of insulin and zinc on intracellular proteins via analysis of an intracellular protein array (see Table 1). a. Mouse skeletal muscle cells. Top panel, control (untreated cells), middle panel, insulin treated cells; bottom panel, $ZnSO_4$ treated cells. Positive and negative controls are shown labelled on the control panel. Significant responses to insulin and $ZnSO_4$ (PRAS40, Akt, ERK1/2 and GSK-3β) are shown. b. Human skeletal muscle cells. Top panel, control (untreated cells), middle panel, insulin treated cells; bottom panel, $ZnSO_4$ treated cells. Positive and negative controls are shown labelled on the control panel. Significant responses to insulin (PRSA40, Akt and ERK1/2) and $ZnSO_4$ (Akt, ERK1/2, GSK-3β and p38) are shown. All positive signaling molecules in the insulin and zinc treated arrays were normalized to their corresponding signaling molecule on the control array (no treatment).

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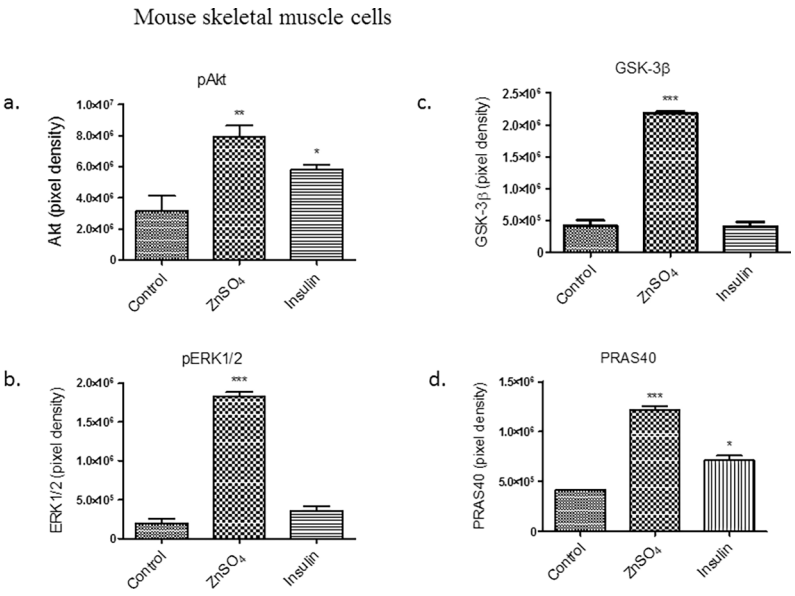

Fig 6. Densitometry results from Fig 5 protein array in mouse skeletal muscle cells. a. pAkt, b. pERK1/2, c. GSK-3β, and d. PRAS40. Control (untreated cells), $ZnSO_4$ and insulin treatments are shown. Differences between the expressions of proteins in cells were determined by one-way ANOVA. The results of 3 independent experiments are presented as a mean ± standard error. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ considered significant when compared to the control.

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that of the control (Fig 7B). The GSK-3β protein level was also significantly higher when treated with zinc ($p < 0.001$) than insulin treatment ($p < 0.05$) and when compared to the control group (Fig 7C). There was no significant difference in the levels of PRAS40 when treated with $ZnSO_4$ and compared to the control (Fig 7D). However, insulin treatment resulted in a significant increase ($p < 0.001$) in PRAS40 compared to the control group. Lastly, $ZnSO_4$ treatment resulted in a significant increase in p38 ($p < 0.001$) over the control group but there was no significance identified during the insulin treatment (Fig 7E). p38, is another signaling factor that plays a significant role in the regulation of glucose transport [32].

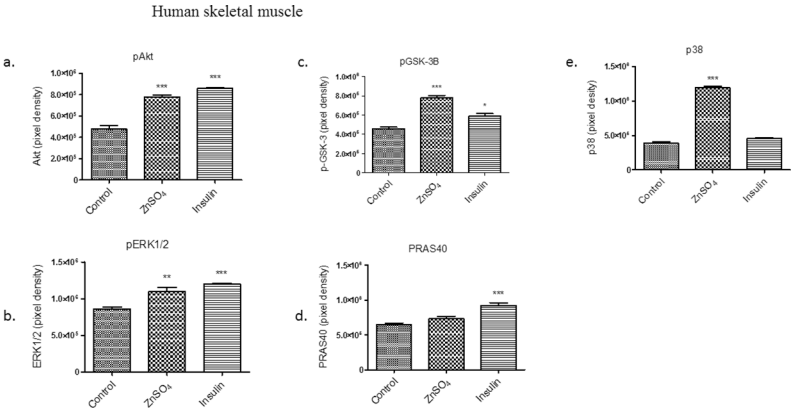

Fig 7. Densitometry results from Fig 5 protein array in human skeletal muscle cells. a. pAkt, b. pERK1/2, c. pGSK-3β, d. PRAS40, and e. p38. Control (untreated cells), $ZnSO_4$ and insulin treatments are shown. Differences between the expressions of proteins in cells were determined by one-way ANOVA. The results of 3 independent experiments are presented as a mean ± standard error. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ considered significant when compared to the control.

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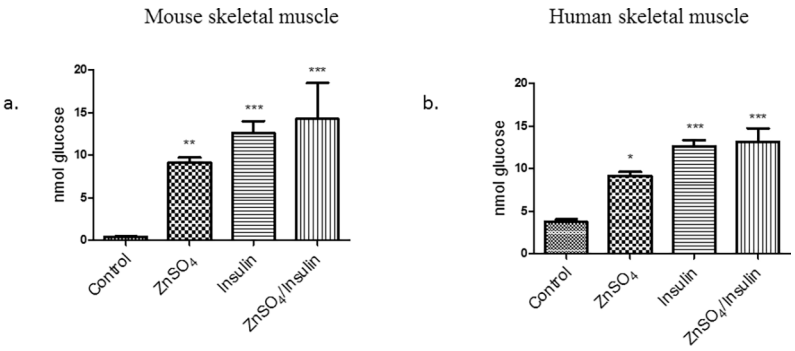

Fig 8. Glucose oxidation assay in the presence of insulin and $ZnSO_4$. a. Mouse skeletal muscle cells, and b. Human skeletal muscle cells. The results of 3 independent experiments are presented as a mean ± standard error. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ considered significant compared with the control (untreated cells).

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Zinc effect of glucose consumption in mouse C2C12 and human skeletal muscle cells

It is clear that zinc affects several cell signaling molecules, analogous to insulin signaling, that are implicated in glucose homeostasis in skeletal muscle. Accordingly, we tested whether zinc could activate glucose oxidation in both mouse and human skeletal muscle cells. Initially we tested glucose oxidation in the presence of insulin treatment, and as expected, insulin significantly induced glucose oxidation in mouse ($p < 0.001$) and human ($p < 0.001$) skeletal muscle cells compared to the untreated cells (Fig 8A and 8B). Similarly, $ZnSO_4$ significantly induced glucose oxidation in both mouse ($p < 0.01$) and human ($p < 0.05$) skeletal muscle cells compared to the untreated control (Fig 8A and 8B). We did not observe a significant additive effect of insulin and $ZnSO_4$ treatment together when compared to $ZnSO_4$ or insulin treatment alone.

Zinc activation of pAKT potentially acts through a functional insulin receptor

Given that we did not observe an additive effect of insulin and $ZnSO_4$ treatment together on glucose oxidation, we sought to determine whether a functional insulin receptor is required to facilitate zinc activation of pAkt. To address this, we utilized C2C12 skeletal muscle cells treated with an insulin receptor tyrosine kinase inhibitor in the presence of insulin or zinc. We observed that 50 μM of the insulin receptor tyrosine kinase inhibitor HNMPA-$(AM)_3$ was sufficient to inhibit insulin-induced pAkt (Fig 9). Similarly, we identified that 25 μM of HNMPA-$(AM)_3$ inhibited zinc-induced pAkt and this was completely abolished at 50 and 100 μM of HNMPA-$(AM)_3$ (Fig 9).

Discussion

In this study, we analyzed the effects of zinc on insulin signaling in mouse and human skeletal muscle cell lines. Insulin signaling is an important and highly conserved regulatory network coordinating metabolism and growth in multicellular organisms [33]. Dysregulation of insulin signaling is common in metabolic disorders. For example, insulin resistance which is a hallmark of type 2 diabetes mellitus [33], is characterized by a reduced insulin-mediated activation of the PI3K/Akt pathway regulating glucose uptake [34]. Several studies have suggested that zinc, as an essential trace element, has a potent ability on glucose handling by promoting beta cell function and insulin sensitivity [35]. Zinc in skeletal muscle accounts for most of the

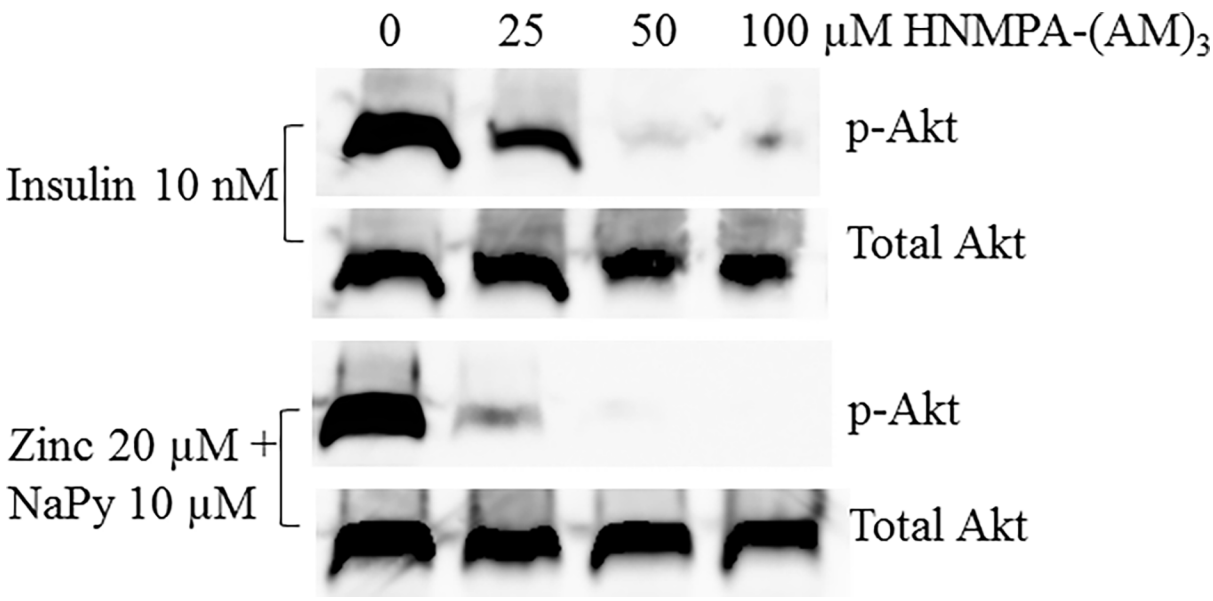

Fig 9. Effect of insulin receptor tyrosine kinase inhibitor HNMPA-$(AM)_3$ on insulin and zinc-induced pAkt. Mouse C2C12 skeletal muscle cells were treated with increasing concentrations (0, 25, 50, and 100 μM) of HNMPA-$(AM)_3$ followed by either treatment with insulin (10 nM) or zinc (20 μM) for 1 hour. Concentrations of HNMPA-$(AM)_3$ are given at 0, 25, 50 and 100 μM. Top panel: insulin-treated measurement of pAkt, Lower panel: zinc-treated measurement of pAkt. Total Akt was used as an internal loading control in both panels and levels of pAKT were normalized to total Akt.

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whole-body zinc (approximately 60%) [36], and as skeletal muscle is the major site of peripheral insulin resistance [37], we investigated the effects of zinc signaling in skeletal muscle cells.

Regarding the participation of zinc in insulin signaling, it is emphasized that this metal ion promotes activation of phosphatidylinositol protein 3-kinase and protein kinase B (Akt), increasing transport of glucose uptake to cells [38]. Our data shows that there is an increase in the phosphorylation of Akt in mouse C2C12 and human skeletal muscle cells treated with zinc, indicating the role of zinc in the induction of Akt phosphorylation. During insulin signaling, the activated insulin receptor kinase, phosphorylates tyrosine residues on insulin receptor substrate 1 (IRS1) and insulin receptor substrate 2 (IRS2) and this subsequently generates a tyrosine phosphorylation cascade to transmit the insulin signal [39]. Our data show that there is an increase in phospho-tyrosine in both mouse and human skeletal muscle cell treated with zinc and suggests that zinc also plays a role in transmitting the insulin signal.

Previous studies have shown that SHP-2 can bind to IRS-1 in response to insulin and modulate receptor tyrosine kinase-mediated signaling [40]. Our data indicates that zinc can also activate SHP-2 in mouse and human skeletal muscle cells. Similarly, studies have shown that the ERK/MAP kinase signaling pathway is an important mediator in insulin responsiveness [41]. According to our results, zinc increases the expression of ERK in both cell lines which might help to improve insulin signaling in skeletal muscle.

Our results have also shown that zinc exhibited insulin-like glucose transporting effects by phosphorylation of key proteins involved in the insulin signaling cascade including GSK-3β. GSK-3β plays several important roles in mammalian cells, and impacts such diverse cellular processes including glycogen synthesis and glucose transport. Overactivity of GSK-3β in skeletal muscle of obese type 2 diabetic humans is associated with an impaired ability of insulin to activate glucose disposal and glycogen synthase [42]. Studies have also demonstrated that inhibition of non-phosphorylated form of GSK-3 can improve insulin-stimulated glucose transport activity through enhanced post-insulin receptor insulin signaling and GLUT-4 glucose

transporter translocation [43]. Accordingly, GSK-3β serine phosphorylation, which is the inactive form of GSK-3β, increased after treating cells with zinc.

Our data indicate that zinc increases PRAS40 in mouse skeletal muscle cells. PRAS40 is a regulator of insulin sensitivity which has an inhibitory effect on mTOR and consequently decreases mTOR expression which is a negative regulator of IRS1 in mouse skeletal muscle cell line. It causes an abundance in IRS1 and enhances insulin-mediated Akt phosphorylation and increases glucose uptake [44].

Activation of p38 is not only dependent on stimulus, but on cell type as well. For example, insulin can stimulate p38 in 3T3-L1 adipocytes, but down-regulates p38 activity in chick forebrain neuron cells [45]. We have reported that the expression of p38 increases in human skeletal muscle cells treated with zinc. Although p38 phosphorylation has been reported to be increased in response to insulin in skeletal muscle from non-diabetic subjects [46], we did not observe this in our experiments. It is not clear why p38 did not respond to insulin in this study. Possibly, the thirty-minute, insulin treatment time-point used on the protein arrays missed the maximum response for p38. For example, treatment of mouse L6 myotubes with 100 nM insulin resulted in a maximum p38 response at ten minutes which declined at fifteen minutes of treatment [47]. Similarly, in rat vascular smooth muscle cells (VSMC), p38 was rapidly activated in the presence of insulin at five minutes and quickly declined to basal levels at fifteen minutes [48]. We also identified that zinc can oxidize glucose in both mouse and human skeletal muscle cells. A previous study in rat adipocytes showed that zinc could enhance the metabolism of glucose which was indicative of enhancement of facilitated glucose transport [49]. While both insulin or zinc could independently activate glucose oxidation, an additive effect of insulin and zinc was not observed. Additionally, we found that inhibition of insulin receptor tyrosine kinase activity abolished the ability of insulin and zinc to activate pAkt and therefore suggests that zinc potentially acts through the insulin signaling pathway. These data are supported by previous studies where co-incubation with zinc and insulin did not enhance glucose consumption in normal L6 cells and suggests that zinc and insulin may act through the same downstream pathways [17]. However, in insulin-resistant L6 myotubes, zinc and insulin together could enhance glucose consumption when compared to insulin treatment alone [17]. These data suggest that disrupted insulin signaling in the insulin resistant L6 cells respond differently to zinc than in normal L6 cells and therefore might provide clues on alternative signaling pathways under zinc stimulation.

Skeletal muscle is of major importance in insulin resistance and T2DM as it accounts for 85% of whole body insulin-dependent glucose uptake and therefore plays a critical role in maintaining systemic glucose metabolism [37]. Defects in glucose metabolism in insulin resistant skeletal muscle include reduced insulin receptor tyrosine phosphorylation [50] decreased Akt phosphorylation [51–52], and impaired GLUT4 translocation [52,53]. These insulin protein targets are also activated by zinc treatment in skeletal muscle [3,17,54].

The ability of zinc to regulate insulin signaling processes suggests that this metal ion might have utility to be targeted experimentally to improve the management and/or treatment of insulin resistance. One well-studied target of the insulin-mimetic effects of zinc is protein tyrosine phosphatase 1B (PTP1B). This phosphatase has a key role in the regulation of insulin action by dephosphorylating the insulin receptor and insulin receptor substrates 1 and 2, thereby inhibiting the insulin signaling cascade [55]. It is well-established that zinc can inhibit PTP1B which affects insulin signaling [18]. Moreover, PTP1B knockout mice are highly sensitive to insulin, have low adiposity, and are protected from diet-induced obesity [56]. These studies highlight the dynamic role of zinc in insulin signaling processes and therefore offers opportunities to be further investigated. Type 2 diabetes mellitus is increasing globally and is approaching pandemic levels. Current strategies for prevention are limited in scope and effectiveness, and the

persistently high prevalence of T2DM confirm the shortfalls of available therapeutic options. The 'window of opportunity' that presents before the loss of β-cell function and the development and progression of T2DM suggests that a strategy aimed at reversing insulin resistance would have major clinical outcomes and benefits for health and wellbeing. From a therapeutic perspective, it is important that the molecular mechanisms of dysfunctional zinc transport and defective zinc compartmentalization are identified in human skeletal muscle cells. Similarly, characterizing how zinc leads to increased glucose homeostasis is paramount to understanding potential intervention strategies where zinc transport could be manipulated experimentally to improve glucose metabolism in patients with insulin resistance or T2DM.

Conclusions

Zinc is an essential metal ion implicated in several biological processes. Dysfunctional zinc signaling is associated with various disease states. We have shown that zinc independently activates insulin signaling pathway proteins in mouse and human skeletal muscle cell lines. The subsequent phosphorylation events associated with insulin signaling and increased glucose oxidation was also mirrored with zinc treatment leading to the supposition that both insulin and zinc are critically important in maintaining glucose homeostasis in skeletal muscle.

Supporting information

S1 Dataset. Data file of mouse and human protein array densitometry showing the average of three-four independent assays in control, zinc treated, and insulin treated skeletal muscle cells.
(XLSX)

S2 Dataset. Data file of mouse and human glucose oxidation assay showing the average of three independent assays in control, zinc treated, insulin treated and zinc plus insulin treatment in skeletal muscle cells.
(XLSX)

Author Contributions

Conceptualization: Shaghayegh Norouzi, John Adulcikas, Sukhwinder Singh Sohal, Stephen Myers.

Data curation: Shaghayegh Norouzi, Stephen Myers.

Formal analysis: Shaghayegh Norouzi, John Adulcikas, Sukhwinder Singh Sohal, Stephen Myers.

Funding acquisition: Stephen Myers.

Investigation: Sukhwinder Singh Sohal, Stephen Myers.

Methodology: Shaghayegh Norouzi, John Adulcikas, Stephen Myers.

Project administration: Shaghayegh Norouzi, Stephen Myers.

Resources: Stephen Myers.

Software: Stephen Myers.

Supervision: Sukhwinder Singh Sohal, Stephen Myers.

Validation: Stephen Myers.

Visualization: Stephen Myers.

Writing – original draft: Shaghayegh Norouzi.

Writing – review & editing: Shaghayegh Norouzi, John Adulcikas, Sukhwinder Singh Sohal, Stephen Myers.

References

1. Højlund K. Metabolism and insulin signaling in common metabolic disorders and inherited insulin resistance. Danish Medical Journal. 2014, 61:B4890–B4890. PMID: 25123125
2. Taghibiglou C, Rashid-Kolvear F, Van Iderstine SC, Le-Tien H, Fantus IG, Lewis GF, et al. Hepatic very low density lipoprotein-ApoB overproduction is associated with attenuated hepatic insulin signaling and overexpression of protein-tyrosine phosphatase 1b in a fructose-fed hamster model of insulin resistance. Journal of Biological Chemistry. 2002, 277:793–803. https://doi.org/10.1074/jbc.M106737200 PMID: 11598116
3. Myers SA, Nield A, Chew G-S, Myers MA. The zinc transporter, Slc39a7 (zip7) is implicated in glycaemic control in skeletal muscle cells. PLoS One. 2013, 8:e79316. https://doi.org/10.1371/journal.pone.0079316 PMID: 24265765
4. Magneson GR, Puvathingal JM, Ray WJ Jr. The concentrations of free Mg^{2+} and free Zn^{2+} in equine blood plasma. The Journal of Biological Chemistry. 1987, 262:11140–11148. PMID: 2956262
5. Kambe T, Tsuji T, Hashimoto A, Itsumura N. The physiological, biochemical, and molecular roles of zinc transporters in zinc homeostasis and metabolism. Physiological Reviews. 2015, 95:749–784. https://doi.org/10.1152/physrev.00035.2014 PMID: 26084690
6. McNulty TJ. Taylor CW. Extracellular heavy-metal ions stimulate Ca^{2+} mobilization in hepatocytes. Biochemical Journal. 1999, 339:555–561. PMID: 10215593
7. Ackland ML, Michalczyk A. Zinc deficiency and its inherited disorders-a review. Genes & Nutrition. 2006, 1:41–49.
8. Rutter GA, Chabosseau P, Bellomo EA, Maret W, Mitchell RK, Hodson DJ, et al. Intracellular zinc in insulin secretion and action: A determinant of diabetes risk? Proceedings of the Nutrition Society. 2016, 75:61–72. https://doi.org/10.1017/S0029665115003237 PMID: 26365743
9. Myers SA. Zinc transporters and zinc signaling: New insights into their role in type 2 diabetes. International Journal of Endocrinology. 2015: http://dx.doi.org/10.1155/2015/167503.
10. Wijesekara N, Dai FF, Hardy AB, Giglou PR, Bhattacharjee A, Koshkin V, et al. Beta cell-specific Znt8 deletion in mice causes marked defects in insulin processing, crystallisation and secretion. Diabetologia. 2010, 53:1656–1668. https://doi.org/10.1007/s00125-010-1733-9 PMID: 20424817
11. Chimienti F, Devergnas S, Favier A, Seve M. Identification and Cloning of a -Cell–Specific Zinc Transporter, ZnT-8, Localized Into Insulin Secretory Granules. Diabetes. 2004, 53:2330–2337. PMID: 15331542
12. Maret W. Zinc in Pancreatic Islet Biology, Insulin Sensitivity, and Diabetes. Preventative Nutrition and Food Science. 2017, 22:1–8.
13. Sladek S, Rocheleau G, Rung J, Dina C, Shen L, Serre D, et al. A genome-wide association study identifies novel risk loci for type 2 diabetes. Nature. 2007, 445:881–885. https://doi.org/10.1038/nature05616 PMID: 17293876
14. May JM, Contoreggi CS. The mechanisms of the insulin-like effects of ionic zinc. Journal of Biological Chemistry. 1982, 257:4362–4368. PMID: 6279634
15. Tang S, Le-Tien H, Goldstein BJ, Shin P, Lai R, Fantus GI. Decreased In Situ Insulin Receptor Dephosphorylation in Hyperglycemia-Induced Insulin Resistance in Rat Adipocytes. Diabetes. 2001, 50:83–90. PMID: 11147799
16. Miranda RE, Dey SC. Effect of chromium and zinc on insulin signaling in skeletal muscle cells. Biological Trace Element Research. 2004, 101:19–36. https://doi.org/10.1385/BTER:101:1:19 PMID: 15516700
17. Wu Y, Lu H, Yang H, Li C, Sang Q, Liu X, et al. Zinc stimulates glucose consumption by modulating the insulin signaling pathway in L6 myotubes: essential roles of Akt–GLUT4, GSK3β and mTOR–S6K1. Journal of Nutritional Biochemistry. 2016, 34:126–135. https://doi.org/10.1016/j.jnutbio.2016.05.008 PMID: 27295130
18. Hasse H, Maret W. Intracellular zinc fluctuations modulates protein tyrosine phosphatase activity in insulin/insulin-like growth factor-1 signaling. Experimental Cell research. 2003, 291:289–298. PMID: 14644152

19. Hasse H, Maret W. Fluctuations of cellular, available zinc modulate insulin signaling via inhibition of protein tyrosine phosphatases. Journal of Trace Elements in Medicine and Biology. 2005, 19:37–42. https://doi.org/10.1016/j.jtemb.2005.02.004 PMID: 16240670

20. Bellomo E, Massarotti A, Hogstrand C, Maret W. Zinc ions modulate protein tyrosine phosphatase 1B activity. Metallomics. 2014, 6:1229–1239. https://doi.org/10.1039/c4mt00086b PMID: 24793162

21. Taylor KM, Vichova P, Jordan N, Hiscox S, Hendly R, Nicholson RI. ZIP7-Mediated Intracellular Zinc Transport Contributes to Aberrant Growth Factor Signaling in Antihormone Resistant Breast Cancer Cells. Endocrinology. 2008, 149:4912–4920. https://doi.org/10.1210/en.2008-0351 PMID: 18583420

22. Taylor KM, Hiscox S, Nicholson RI, Hogstrand C, Kille P. Protein Kinase CK2 Triggers Cytosolic Zinc Signaling Pathways by Phosphorylation of Zinc Channel ZIP7. Science Signaling. 2012, 210: ra11.

23. Nimmanon T, Ziliotto S, Morris S, Flanagan L, Taylor KM. Phosphorylation of zinc channel ZIP7 drives MAPK, PI3K and mTOR growth and proliferation signaling. Metallomics. 2017, 9, 471–481. https://doi.org/10.1039/c6mt00286b PMID: 28205653

24. Vardatsikos G, Pandey NR, Srivastava AK. Insulino-mimetic and anti-diabetic effects of zinc. Journal of Inorganic Biochemistry. 2013, 120:8–17. https://doi.org/10.1016/j.jinorgbio.2012.11.006 PMID: 23266931

25. Mahmood T, Yang PC. Western blot: Technique, theory, and trouble shooting. N Am J Med Sci. 2012, 4:429–434. https://doi.org/10.4103/1947-2714.100998 PMID: 23050259

26. Ijuin T, Takenawa T. Skip negatively regulates insulin-induced Glut4 translocation and membrane ruffle formation. Molecular and Cellular Biology. 2003, 23:1209–1220. https://doi.org/10.1128/MCB.23.4.1209-1220.2003 PMID: 12556481

27. Zhang W, Thompson BJ, Hietakangas V, Cohen SM. MAPK/ERK signaling regulates insulin sensitivity to control glucose metabolism in drosophila. PLoS Genet, 2011. 7:e1002429. https://doi.org/10.1371/journal.pgen.1002429 PMID: 22242005

28. Mannell H, Krotz F. The role of SHP-2 in cell signalling and human disease. Current Enzyme Inhibition. 2007, 3:264–272.

29. Hançer NJ, Qiu W, Cherella C, Li Y, Copps KD, White MF. Insulin and metabolic stress stimulate multisite serine/threonine phosphorylation of insulin receptor substrate 1 and inhibit tyrosine phosphorylation. Journal of Biological Chemistry. 2014, 289:12467–12484. https://doi.org/10.1074/jbc.M114.554162 PMID: 24652289

30. Henriksen EJ, Dokken BB. Role of glycogen synthase kinase-3 in insulin resistance and type 2 diabetes. Current Drug Targets. 2006, 7:1435–1441. PMID: 17100583

31. Wiza C, Nascimento EB, Ouwens DM. Role of PRAS40 in Akt and mTOR signaling in health and disease. American Journal of Physiology-Endocrinology and Metabolism. 2012, 302:E1453–E1460. https://doi.org/10.1152/ajpendo.00660.2011 PMID: 22354785

32. Koistinen HA, Chibalin A, Zierath J. Aberrant p38 mitogen-activated protein kinase signalling in skeletal muscle from type 2 diabetic patients. Diabetologia. 2003, 46:1324–1328. https://doi.org/10.1007/s00125-003-1196-3 PMID: 12937895

33. Zhang W, Thompson BJ, Hietakangas V, Cohen SM. MAPK/ERK signaling regulates insulin sensitivity to control glucose metabolism in drosophila. PLoS Genetics. 2011, 7:e1002429 https://doi.org/10.1371/journal.pgen.1002429 PMID: 22242005

34. Wiza C, Nascimento EB, Ouwens DM. Role of PRAS40 in Akt and mTOR signaling in health and disease. American Journal of Physiology-Endocrinology and Metabolism. 2012, 302:E1453–E1460. https://doi.org/10.1152/ajpendo.00660.2011 PMID: 22354785

35. Islam MR, Attia J, Ali L, McEvoy M, Selim S, Sibbritt D, et al. Zinc supplementation for improving glucose handling in pre-diabetes: A double blind randomized placebo controlled pilot study. Diabetes Research and Clinical Practice. 2016, 115:39–46. https://doi.org/10.1016/j.diabres.2016.03.010 PMID: 27242121

36. Hara T, Takeda T, Takagishi T, Fukue K, Kambe T, Fukada T. Physiological roles of zinc transporters: molecular and genetic importance in zinc homeostasis. Journal of Physiological Science. 2017, 67:283–301.

37. Peppa M, Koliaki C, Nikolopoulos P, Raptis SA. Skeletal Muscle Insulin Resistance in Endocrine Disease. 2010, https://doi.org/10.1155/2010/527850 PMID: 20300436

38. Barthel A, Ostrakhovitch EA, Walter PL, Kampkotter A, Klotz L-O. Stimulation of phosphoinositide 3-kinase/Akt signaling by copper and zinc ions: Mechanisms and consequences. Archives of Biochemistry and Biophysics. 2007, 463:175–182. https://doi.org/10.1016/j.abb.2007.04.015 PMID: 17509519

39. Hançer NJ, Qiu W, Cherella C, Li Y, Copps KD, White MF. Insulin and metabolic stress stimulate multisite serine/threonine phosphorylation of insulin receptor substrate 1 and inhibit tyrosine

phosphorylation. Journal of Biological Chemistry. 2014, 289:12467–12484. https://doi.org/10.1074/jbc.M114.554162 PMID: 24652289

40. Noguchi T, Matozaki T, Horita K, Fujioka Y, Kasuga M. Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation. Molecular and Cellular Biology. 1994, 14:6674–6682. PMID: 7935386

41. Zhang W, Thompson BJ, Hietakangas V, Cohen SM. MAPK/ERK signaling regulates insulin sensitivity to control glucose metabolism in drosophila. PLoS Genetics, 2011. 7:e1002429. https://doi.org/10.1371/journal.pgen.1002429 PMID: 22242005

42. Henriksen EJ, Dokken BB. Role of glycogen synthase kinase-3 in insulin resistance and type 2 diabetes. Current Drug Targets. 2006, 7:1435–1441. PMID: 17100583

43. Nikoulina SE, Ciaraldi TP, Mudaliar S, Carter L, Johnson K, Henry RR. Inhibition of Glycogen Synthase Kinase 3 Improves Insulin Action and Glucose Metabolism in Human Skeletal Muscle. Diabetes. 2002, 51:2190–2198. PMID: 12086949

44. Wiza C, Chadt A, Blumensatt M, Kanzleiter T, Herzfeld De Wiza D, Horrighs A, et al. Over-expression of PRAS40 enhances insulin sensitivity in skeletal muscle. Archives of Physiology and Biochemistry. 2014, 120:64–72. https://doi.org/10.3109/13813455.2014.894076 PMID: 24576065

45. Zarubin T, Jiahuai H. Activation and signaling of the p38 map kinase pathway. Cell Research 2005, 15:11–18. https://doi.org/10.1038/sj.cr.7290257 PMID: 15686620

46. Koistinen HA, Chibalin A, Zierath J. Aberrant p38 mitogen-activated protein kinase signalling in skeletal muscle from type 2 diabetic patients. Diabetologia. 2003, 46:1324–1328. https://doi.org/10.1007/s00125-003-1196-3 PMID: 12937895

47. Niu W, Huand C, Nawaz Z, Levy M, Somwar R, Li D, et al. Maturation of the Regulation of GLUT4 Activity by p38 MAPK during L6 Cell Myogenesis. The Journal of Biological Chemistry. 2003, 278:17953–17962. https://doi.org/10.1074/jbc.M211136200 PMID: 12637564

48. Igarashi M, Yamaguchi H, Hirata A, Daimon M, Tominaga M, Kato T. Insulin activates p38 mitogen-activated protein (MAP) kinase via a MAP kinase kinase (MKK) 3/MKK 6 pathway in vascular smooth muscle cells. European Journal of Clinical Investigation. 2000, 30:668–677. PMID: 10964158

49. May JM, Contoreggi CS. The mechanism of the insulin-like effects of ionic zinc. The Journal of Biological Chemistry. 1982, 257:4362–4368. PMID: 6279634

50. Abdul-Ghani MA, DeFronzo RA. Pathogenesis of Insulin Resistance in Skeletal Muscle. Journal of Biomedicine and Biotechnology. 2010, https://doi.org/10.1155/2010/476279 PMID: 20445742

51. Feng X-T, Wang T-Z, Leng J, Chen Y, Liu J-B, Liu Y, et al. Palmitate Contributes to Insulin Resistance through Downregulation of the Src-Mediated Phosphorylation of Akt in C2C12 Myotubes. Bioscience, Biotechnology, and Biochemistry. 2012, 76:1356–1361. https://doi.org/10.1271/bbb.120107 PMID: 22785470

52. Ding H, Heng B, He W, Shi L, Lai C, Xiao L, et al. Chronic reactive oxygen species exposure inhibits glucose uptake and causes insulin resistance in C2C12 myotubes. Biochemical and Biophysical Research Communications. 2016, 478:798–803. https://doi.org/10.1016/j.bbrc.2016.08.028 PMID: 27501754

53. Krook A, Bjomholm M, Galuska D, Jiang XJ, Fahlman R, Myers MG Jr, et al. Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. Diabetes. 2000, 49:284–292. PMID: 10868945

54. Ohashi K, Nagata Y, Wada E, Zammit PS, Shiozuka M, Matsuda R. Zinc promotes proliferation and activation of myogenic cells via the PI3K/Akt and ERK signaling cascade. Experimental Cell Research. 2015, 333:228–237. https://doi.org/10.1016/j.yexcr.2015.03.003 PMID: 25773777

55. Koren S. Inhibition of the protein tyrosine phosphatase PTP1B: potential therapy for obesity, insulin resistance and type-2 diabetes mellitus. Best Practice & Research Clinical Endocrinology & Metabolism. 2007, 21:621–640.

56. Klaman LD, Boss O, Peroni OD, Kim JK, Martino JL, Zabolotny JM, et al. Increased Energy Expenditure, Decreased Adiposity, and Tissue-Specific Insulin Sensitivity in Protein-Tyrosine Phosphatase 1B-Deficient Mice. Molecular and Cellular Biology. 2000, 20:5479–5489. PMID: 10891488

CHAPTER 4

INVESTIGATING THE EFFECTS OF ZINC ON GLUT4 AND GLUCOSE HOMEOSTASIS

4.1. Background.

Glut4 belongs to a 12-member family of glucose transporters and is largely expressed in insulin-sensitive tissues that require glucose such as adipose, skeletal muscle, and cardiac muscle tissue (1). Glut4 contains unique amino acid sequence motifs in the N- and C-terminal cytoplasmic regions that direct characteristic membrane translocation capability (1). Glut4 is stored in the basal state in intracellular compartments including the trans-Golgi network (TGN) and small tubular vesicles, which contrasts with other glucose transporters that are constitutively found in the plasma membrane. In response to insulin and other stimuli, Glut4 is mobilised from its intracellular location and translocated to the outer plasma membrane to facilitate glucose entry into the cell (2).

Insulin-dependent Glut4 mobilisation and membrane translocation is a multistep process. Following insulin targeting the insulin receptor in adipose and skeletal muscle, Glut4 vesicles are mobilised from their intracellular storage compartment and sequestered to the plasma membrane. Here Glut4 is docked on the plasma membrane through an interaction with a membrane targeting-SNARE complex (SNAREs are the fundamental cellular machinery that mediates membrane fusion (3). Once docked, Glut4 vesicles contact and fuse with the plasma membrane to enable glucose influx. Therefore, one of the major roles of insulin is uptake of glucose in muscle and adipose tissue through the translocation of Glut4 to the plasma membrane (4).

The transport of glucose in tissues that are sensitive to insulin has received considerable attention based on the critical importance of whole body glycaemic control and maintenance in the adult (5, 6). The transport of glucose uptake in insulin-sensitive tissues such as adipose and skeletal muscle is a rate-limiting step under most conditions (1).

We have previously shown that zinc could enhance glucose oxidation in both mouse and human skeletal muscle cells (7). We hypothesised that the increased glucose oxidation in skeletal muscle cells treated with zinc is, at least partially, due to the increased Glut4 translocation to the cell membrane and increased glucose uptake by Glut4 transporters. This hypothesis is based on the following experimental evidence: 1) zinc stimulates the phosphorylation of the β subunit of the insulin receptor and promotes activation of phosphatidylinositol protein 3-kinase and protein kinase B (known as Akt), increasing transport of glucose inside cells (8); 2) zinc induces

the translocation of Glut4 in a dose-dependent manner in L6 skeletal muscle myotubes and this was augmented in the presence of insulin (9); and 3) zinc significantly increased glucose transport *via* PI3K and Akt in 3T3-L1 fibroblasts and adipocytes (10).

Accordingly, this study aimed to test the ability of zinc to translocate Glu4 to the cell surface of skeletal muscle cells utilising western blotting and confocal microscopy. We identified that zinc treatment significantly increased the levels of the Glut4 protein in C2C12 skeletal muscle cells. Treatment of C2C12 cells with insulin or zinc and the subsequent subcellular fractionation of cytosolic versus membrane components, resulted in the majority of Glu4 expression confined to the membrane component. However, control C2C12 cells not treated with insulin or zinc also showed membrane localised Glu4 expression. Similarly, confocal microscopy of Glut4 following stimulation of C2C12 cells with insulin or zinc resulted in cytosolic localisation of Glut4. This was also not different from the control cells not treated with insulin or zinc. These results suggest there was potentially some experimental design and process issues, or C2C12 cells are somewhat insensitive to insulin and/or lack functional Glut4 machinery that is required to translocate to the outer plasma membrane.

4.2. Methodology.

4.2.1. Cell culture.

C2C12 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L D-glucose, L-glutamine and 110 mg/L sodium pyruvate with 10% fetal calf serum and 5% penicillin-streptomycin-glutamine. The cells were incubated at 37°C and 5% CO2 in a humidified atmosphere.

Passaging and Culturing Adherent Cells: Proliferating C2C12 cells were washed using a 37°C balanced salt solution without calcium and magnesium (approximately 2 mL per 10 cm^2 culture surface area). Pre-warmed trypsin (Thermo fisher, Australia) (37°C), was added to the side of the flask (Thermo fisher, Australia) (approximately 0.5 mL per 10 cm^2 to cover the cell layer) and the culture vessel was incubated at room temperature for approximately two minutes. When approximately 90% of the cells were detached, the equivalent of two volumes (twice the volume used for the dissociation reagent) of pre-warmed complete DMEM growth medium was added. The medium was dispersed by pipetting over the cell layer surface several times.

The cells were transferred to a 15-mL conical tube (Thermo fisher, Australia) and centrifuged at 200 × *g* for 5 minutes at room temperature. The cell pellet was resuspended in a pre-warmed complete DMEM growth medium and the cells were seeded into the culture vesicle at a density of only 5.0 x10^3 viable cells/cm^2 and incubated at 37°C.

4.2.2. Protein Extraction and Western Blot.

Total cellular protein was extracted by scraping cells with RIPA buffer (Thermo Fisher, Victoria, Australia) containing a protease and phosphatase inhibitor cocktail (Sigma, New South Wales, Australia) and samples were placed on ice for 1 hour with constant vortexing every 10 minutes. The samples were centrifuged at 13,000 rpm for 5 minutes and the supernatant was collected. Total protein concentration was determined using a BCA kit as per manufacturer's instructions (Thermo Fisher). Thirty micrograms of total soluble protein were loaded in protein loading dye (5X solution of 250 mM Tris-HCl, pH 6.8, 10% SDS, 30% (v/v) Glycerol, 10 mM DTT, 0.05% (w/v) Bromophenol Blue). Proteins were resolved on a 4–15% SDS-PAGE gradient gel (BioRad, New South Wales, Australia) and transferred to a nitrocellulose membrane (Thermo Fisher). The membrane was blocked for 1 hour in TBS-Tween 20 buffer (50 mmol/L Tris-Cl, pH 7.6, 150 mmol/L NaCl and 0.1 % Tween 20) containing 5 % (w/v) casein. The membrane was then incubated overnight with the primary antibody in 0.5% casein in TBS-Tween buffer. Following primary antibody incubation, the membrane was washed four times for 10 minutes in TBS-Tween buffer. Then, the membrane was incubated with the secondary antibody for 1 hour at room temperature in 0.5% casein in TBS-Tween buffer. Following this, the membrane was washed four times for 10 minutes in TBS-Tween buffer. Immunoreactive signals were detected using SuperSignal West Femto kit (Thermo Fisher) and visualized by a UVITEC Alliance digital imaging system (Thermo Fisher). To assess protein loading consistency, the membranes were stripped with Restore Plus Western Blot Stripping Buffer (Thermo Fisher) by incubating the membrane in the buffer for 15 minutes at room temperature on an end-over-end shaker. The membranes were subsequently washed in TBS-Tween buffer and blocked with 5% skim milk before adding the appropriate primary antibody (11). The subsequent blots were then processed as outlined above.

4.2.3. Subcellular fractionation of cultured cell samples.

To establish the subcellular fractionation assay, C2C12 skeletal muscle cells were cultured in six well plates to reach 100% confluence. After three hours of serum-free condition, the cells were treated with 10 nM insulin, 20 μM of $ZnSO_4$ in the presence of 10 μM sodium pyrithione (NaPy) (Sigma), and DMEM alone (control) for 15 minutes. The cytosolic, membrane and nuclear subcellular fractionation of skeletal muscle cells was performed using Qproteome Cell Compartment Kit (QIAGEN, Victoria, Australia). By sequential addition of several different extraction buffers to the C2C12 cell pellet, proteins in the membrane and cytoplasm of skeletal muscle cells were selectively isolated **(Table 4.1)**.

Table 4.1. Subcellular protein fractionation from cellular components.

Buffer	Isolated proteins
Extraction buffer CE1	Cytosol
Extraction buffer CE2	Membranes

Extraction Buffer CE1 was added to cells to disrupt the plasma membrane without solubilising it, resulting in the isolation of cytosolic proteins. Plasma membranes and compartmentalised organelle membranes were then pelleted by centrifugation. The pellet was then resuspended in Extraction Buffer CE2 to solubilise the plasma membrane and all organelle membranes except the nuclear membrane. After solubilisation, the sample was centrifuged. The subsequent supernatant contained all membrane proteins and proteins from the lumen of organelles.

The procedure is as follows;

A cell suspension containing 5 x 10^6 cells was transferred into a 15 mL conical tube and centrifuged at 500 x *g* for 10 minutes at 4°C. The supernatant was removed and discarded. The cell pellet was resuspended in 2 mL ice-cold PBS by pipetting up and down with a 1 mL pipette tip and the cell suspension was transferred into a microcentrifuge tube. Cells were then pelleted by centrifuging at 500 x *g* for 10 minutes at 4°C. The supernatant was removed and discarded. This step was repeated. A Protease Inhibitor Solution (PIS) (100x) was added to the Extraction Buffer CE1. The cell pellet was resuspended in 1 mL ice-cold Extraction Buffer CE1 by pipetting up and down using a 1 mL pipette tip, incubated for 10 minutes at 4°C on an end-over-end shaker. The lysate was centrifuged at 1000 x *g* for 10 minutes at 4°C. The supernatant containing cytosolic proteins was transferred into a fresh microcentrifuge tube and stored on

ice. A PIS (100x) was added to Extraction Buffer CE2. The pellet was resuspended in 1 mL ice-cold Extraction Buffer CE2 by pipetting up and down using a 1mL pipette tip and incubated for 30 minutes at 4°C on an end-over-end shaker. The subsequent suspension was centrifuged at 6000 x *g* for 10 minutes at 4°C. The supernatant containing the membrane proteins was transferred into a fresh microcentrifuge tube and stored on ice.

4.2.4. Transformation of E. *coli* cells with GFP vector.

Plasmid Green Fluorescent Protein (GFP) DNA (EX-Mm19596-M03, GeneCopoeia, United BioResearch Products, New South Wales, Australia) **(Figure 4.1)** was transformed into high-efficiency E. *coli* bacterial cells (Alpha-select chemically competent cells, Bioline, New South Wales, Australia). Briefly, 5 µL of DNA solution was added to 100 µL cells suspension ($\geq 10^9$cfu/µg) and incubated on ice for 30 minutes. Then, the tube was placed in a 42°C water bath for 45 seconds. The tube was then placed on ice for 2 minutes. The transformation reaction was diluted to 1 mL by the addition of 900 µL SOC medium (2% Tryptone, 0.5% Yeast Extract, 0.4% Glucose, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$) and aerated at 200 rpm for 60 minutes at 37°C. The transformation mixture was plated on LB agar plates containing appropriate antibiotic (Ampicillin) and incubated overnight at 37°C.

Bacterial colonies were harvested, and plasmids were isolated using an ISOLATE II plasmid Midi kit as per the manufacturer's instructions (Bioline). Briefly, overnight bacterial culture was harvested by centrifuging at 6000 x *g* for 15 minutes at 4°C. The bacterial pellet was resuspended in 4 mL Buffer P1 followed by the addition of 4 mL of Buffer P2, was mixed thoroughly by vigorously inverting 4-6 times and incubated at room temperature for 5 minutes. Four mL of prechilled Buffer P3 was added and mixed by vigorously inverting 4-6 times and incubated on ice for 15 minutes and centrifuged at 20000 x *g* for 30 minutes at 4°C. The supernatant was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. DNA was eluted with 5 mL Buffer QF and precipitated by adding 3.5 mL room-temperature isopropanol and centrifuged at 15000 x *g* for 30 minutes. The DNA pellet was washed with 2 mL room-temperature 70% ethanol and centrifuged at 15000 x *g* for 10 minutes. The DNA pellet was then air-dried for 10 minutes and redissolved in 10 mM Tris-Cl, pH 8.5. Restriction digest analysis using the enzymes EcoRI and XhoI were used to determine the correct insertion of the Glut4-GFP plasmid (**Figure 4.1**). Plasmids were transfected into skeletal muscle cells

using Lipofectamine 3000 reagent (Thermo Fisher) as per manufacturer's instructions. Briefly, skeletal muscle cells were cultured in 8-well chambers. Once the cells have reached 70% confluence, approximately 1 µg of plasmid was mixed with the Lipofectamine 3000 in DMEM and placed on the cells for 24 hours.

Vector Features

Promoter	CMV
Host cell	Mammalian
Bacterial selection antibiotics	Ampicillin/Neomycin
Mammalian selection marker	Yes
Tag	C-GFP

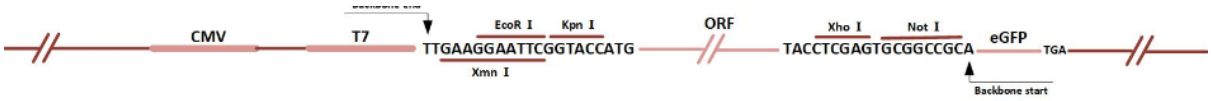

Figure 4.1. GFP EX-Mm19596-M03 Expression Clone (CMV Promoter) and Restriction Enzyme Information. GFP is driven by a CMV promoter and clones were isolated based on screening for ampicillin resistance. Restriction sites for *EcoR1, Kpn1, Xho1* and *Not1* are given for orientation of the open reading frame (ORF).

4.2.5. Confocal Microscopy

Confocal microscopy was performed following the methods described previously (12) with minor modifications. C2C12 skeletal muscle cells were grown in 8-well chamber slides at low density (1 x10^5 cells/well) and serum starved for 3 hours before incubation in the presence and absence of 100 nM insulin for 15 minutes, or in the presence and absence of 20 µM zinc + ionophore 10 µM for 15 minutes. Cells were then fixed using 4% (vol/vol) formaldehyde in PBS at room temperature for 10 minutes, quenched with 50 mM NH_4Cl for 15 minutes and blocked for 1 hour with 10% FCS serum. Cells were incubated with anti-Glut4 primary antibody (Abcam, Victoria, Australia), diluted in blocking solution at 1:200 overnight at 4˚C, followed by three washes of 5 minutes with PBS. Cells were then incubated with Goat Anti-Rabbit IgG H&L (FITC) secondary antibody (1:1000) (Abcam) for 30 minutes in a dark room. After three washes with PBS, slides were mounted with Prolong Diamond with DAPI (Thermo

Fisher), left to cure overnight and imaged using an Olympus IX83 confocal microscope (Olympus, Victoria, Australia).

To investigate Glut4 translocation using the plasmid encoding Glut4-GFP following treatments with zinc or insulin, cells were placed on ice immediately and washed three times with ice-cold Krebs-Ringer HEPES buffer [KRBH; 120 mM NaCl, 25 mM HEPES, 4.6 mM KCl, 1 mM MgSO4, 1.2 mM KH2PO4 and 1.9 mM CaCl2 (pH 7.4)]. Slides were mounted with Prolong Gold with DAPI from Thermo fisher, cured overnight and imaged as described above.

4.2.6. Statistical analysis

Protein expression data from western blots were quantified by ImageJ quantification normalised to Gapdh (Genesearch, Queensland, Australia). Microscopy images were analysed by Prism 7 software and an ANOVA with Dunnett's pair-wise comparisons. All data is normalised to the control mean represented as control=100 ± standard deviation (SD) with *, ** and *** indicating $p<0.05$, $p<0.01$ and $p<0.001$ statistical differences compared to control, respectively.

4.3. Results.

4.3.1. Zinc treatment increases Glut4 protein levels in normal C2C12 cells.

To measure the protein expression of Glut4 in the presence of zinc, C2C12 skeletal muscle cells were treated with 20 μM $ZnSO_4$ in the presence of 10 μM NaPy over 16 hours and subsequent western blots were performed on immunoreactive Glut4 **(Figure 4.2)**. It was observed that zinc treatment resulted in a significant increase in the protein expression of Glut4 (total cellular Glut4 content) after 2 ($p<0.01$), 4 ($p<0.01$) and 16 ($p<0.001$) hours compared to the zero control cells (**Figure 4.2**).

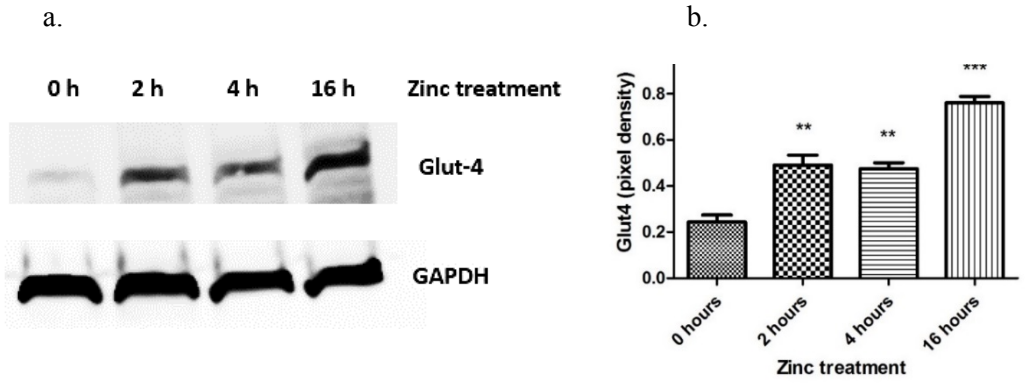

Figure 4.2. Western blot results of Glut4 protein expression (a) and densitometry results (b) in mouse C2C12 skeletal muscle cells treated with 20 µM zinc and 10 µM NaPy over 16 hours. a. Time is shown in hours from 0, 2, 4 and 16 and Gapdh was used as an internal loading control. **b.** Data are presented as means ±SD (n=3 independent experiments) and were analysed using one-way ANOVA followed by post-test Turkey. (Glut4: glucose transporter 4, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase).

4.3.2. Expression of cytosolic versus membrane Glut4 in mouse skeletal muscle cells treated with insulin or zinc.

Insulin acts within minutes to mobilise Glut4 storage vesicles to translocate Glut4 to the plasma membrane to enhance glucose uptake (13). To determine whether zinc can also mobilise Glut4 to the cell surface, western blots were performed on cytosolic and membrane proteins extracted from C2C12 skeletal muscle cells treated with zinc or insulin **(Figure 4.3)**. There was a clear difference in the expression of Glut4 in the membrane fraction, compared to the cytosolic fraction in C2C12 zinc treated cells (**Figure 4.3a**). It appeared that the control C2C12 cells (not treated with insulin or zinc) also showed a clear difference in the membrane fraction versus the cytosolic fraction (**Figure 4.3b**). Following treatment of C2C12 skeletal muscle cells with 100 nM insulin, Glut4 expression was abundant in the membrane fraction but not the cytosolic fraction (**Figure 4.3c**). Gapdh was used as a control as this enzyme is localised to the cytosolic fraction. There is a clear differentiation between the expression levels of Gapdh in the cytosol versus the membrane fraction **(Figure 4.4a-c)**.

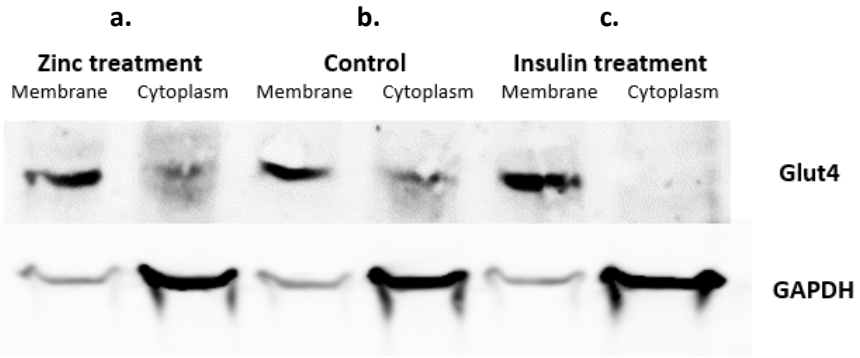

Figure 4.3. Western blot result of Glut4 expression in subcellular fractionated C2C12 cells. a. Cells were treated with 20 μM $ZnSO_4$ in the presence of 10 μM NaPy for 15 minutes. **b.** Untreated control cells. **c.** Cells treated with 10 mM insulin for 15 minutes. Gapdh was used as a control protein specific to cytoplasm fraction. This is a representative blot from three independent experiments. (Glut4: glucose transporter 4, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase).

4.3.3. The localisation of Glut4 in the presence of insulin or zinc in the cytoplasm or membrane of C2C12 cells by confocal microscopy.

Glut4 translocation to the cell surface is the final step of the insulin-stimulated glucose uptake process, and the quantity of Glut4 at the cell surface is the rate-limiting step of glucose disposal (9). To determine if Glut4 could be mobilised by zinc to the cell surface in C2C12 skeletal muscle cells, confocal microscopy was performed. C2C12 cells were treated for 15 minutes with 20 μM $ZnSO_4$ in the presence of 10 μM NaPy, 10 nM insulin as a positive control and neither zinc nor insulin as a negative control. It was anticipated that C2C12 cells treated with insulin would show an increase in the mobilisation of cytosolic Glut4 to the plasma membrane. It was identified that in the presence of insulin, Glut4 was localised to the cell membrane however, the control cells not treated with insulin also showed Glut4 membrane-bound protein **(Figure 4.4b and 4.4c)**. Similarly, treatment of C2C12 cells with zinc resulted in Glut4 being localised to the cell membrane, however, this was not different from the control group of cells not treated with zinc **(Figure 4.4b and 4.4d)**.

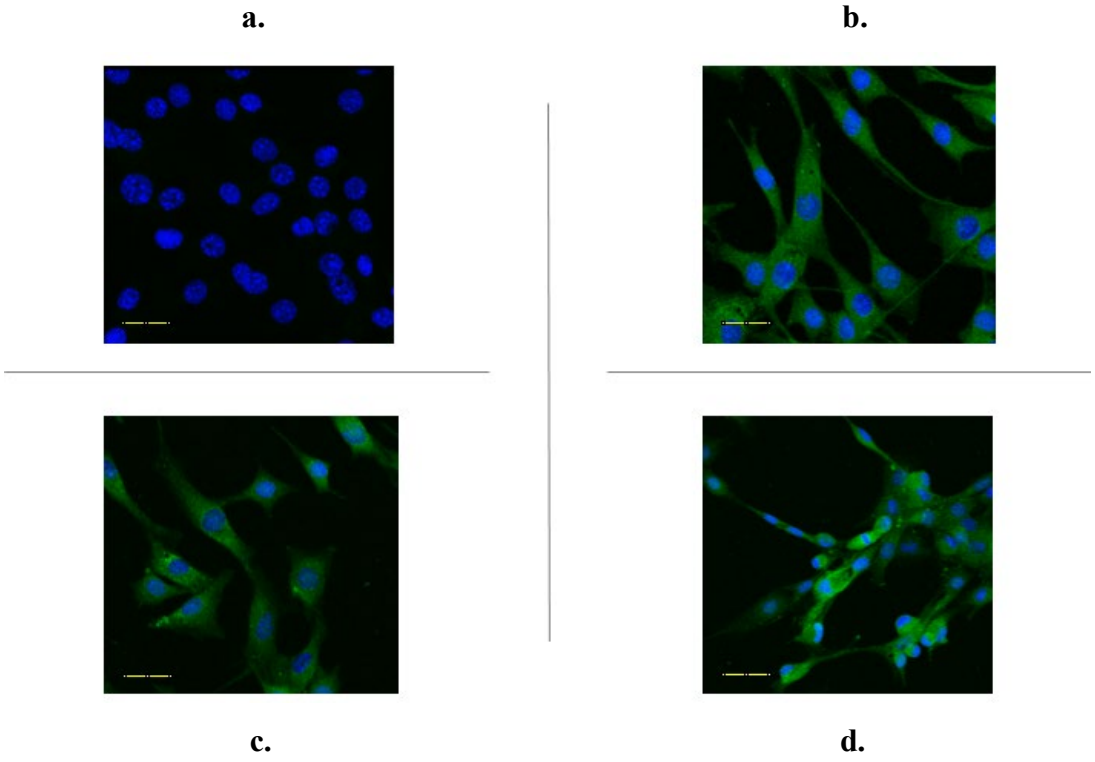

Figure 4.4. Glut4 expression of C2C12 cells quantified by confocal microscopy. **a**. Control cells (without secondary antibody); **b**. Control cells not treated with insulin or zinc; **c**. Cells treated with 10 mM insulin for 15 minutes; and **d**. Cells treated with 20 µM $ZnSO_4$ in the presence of 10 µM NaPy for 15 minutes. Immunofluorescence staining was used to determine the subcellular localization of Glut4. The scale bars (yellow line) represent 10 µm.

As shown in **Figure 4.5**, insulin (c) or zinc treatment (d) did not significantly induced Glut4 membrane translocation over that of control (b) in C2C12 cells.

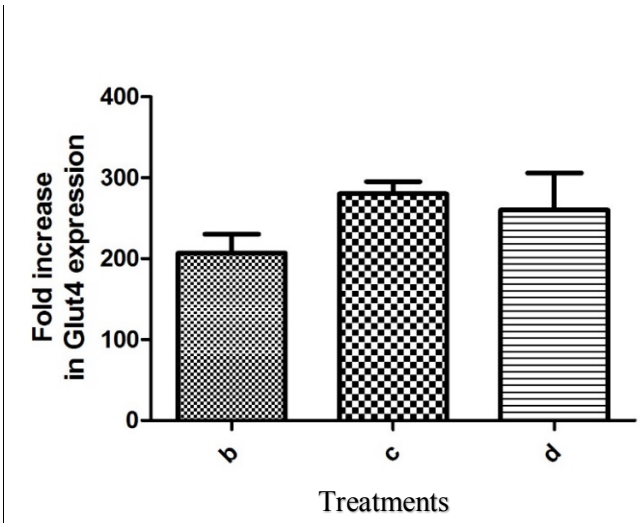

Figure 4.5. Confocal microscopy of Glut4 expression in C2C12 cells. **b**. Control cells not treated with insulin or zinc; **c**. Cells treated with 10 mM insulin for 15 minutes; and **d**. Cells treated with 20 µM $ZnSO_4$ in the presence of 10 µM NaPy for 15 minutes. N=7 cells from three randomly selected images and three independent assays were taken under 63X oil-emersion.

To further investigate Glut4 mobilisation to the cell surface in C2C12 cells by confocal microscopy, a plasmid-based system encoding Glut4-GFP was used. Following treatments with insulin (**Figure 4.6c**) or zinc (**Figure 4.6d**), C2C12 skeletal muscle cells did not show any changes in the mobilisation of cytosolic Glut4 to the plasma membrane compare to the control **(Figure 4.6b)**.

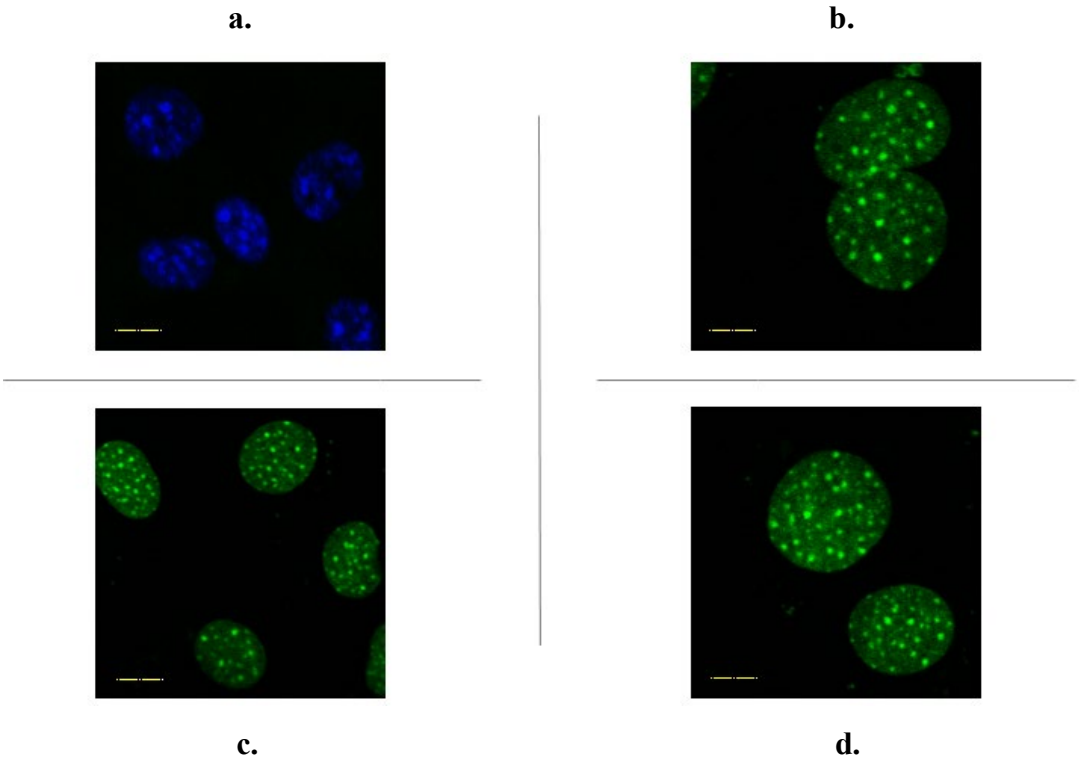

Figure 4.6. Glut4-GFP expression of C2C12 cells quantified by confocal microscopy. a. Control (without secondary antibody); **b**. Control cells not treated with insulin or zinc; **c**. Cells treated with 10 mM insulin for 15 minutes; and **d**. Cells treated with 20 µM $ZnSO_4$ in the presence of 10 µM NaPy for 15 minutes. The scale bars represent 10 µm.

As shown in **Figure 4.7**, there was no significant difference in Glut4 localisation in C2C12 cells treated with insulin or zinc when compared to the control.

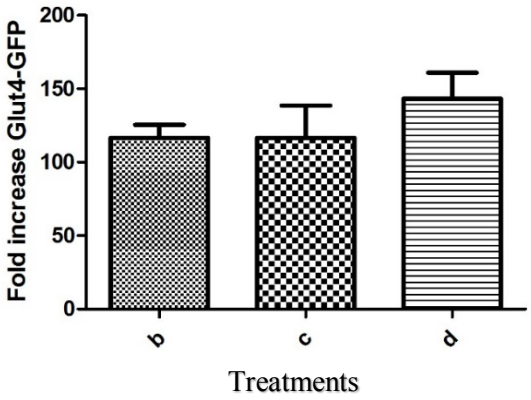

Figure 4.7. Confocal microscopy results of Glut4-GFP expression in C2C12 cells. **b**. Control cells not treated with insulin or zinc; **c**. Cells treated with 10 mM insulin for 15 minutes; and **d**. Cells treated with 20 μM $ZnSO_4$ in the presence of 10 μM NaPy for 15 minutes. N=7 cells from three randomly selected images and three independent assays taken under 63X oil-emersion.

4.4. Conclusion

The glucose transporter, Glut4, facilitates the transport of glucose in skeletal muscle and adipose tissue (14). The mobilisation of Glut4 in the presence of insulin is a well-established mechanism that allows glucose to enter skeletal muscle cells and be utilised for metabolic processes including energy expenditure and glycogen storage (15). Upon digestion of food, glucose enters the blood stream and increases blood glucose levels. In response to this increase in blood glucose, insulin is secreted from the pancreatic beta-cells. Secreted insulin increases peripheral glucose utilisation and decreases hepatic gluconeogenesis. These set of events restore the blood glucose level to a physiological range within two to three hours following a meal. In IR and T2DM, this process is compromised and therefore fails to maintain glucose homeostasis (14).

It has previously been reported that zinc exerts insulin-like effects by upregulating Akt phosphorylation and Glut4 translocation to the plasma membrane in normal rat L6 skeletal muscle myotubes (9). Previous investigations showed that zinc has the capacity to increase Akt phosphorylation leading to GSK-3β activation in addition to increasing total glucose consumption in skeletal muscle cells (7). This calls into question what effects zinc could elicit on the translocation of the glucose transporter, Glut4, in skeletal muscle? Understanding the

molecular mechanisms of zinc action has been of major interest since the discovery that zinc stimulates glucose oxidation in C2C12 skeletal muscle cells (7). Although the molecular mechanisms have yet to be elucidated, it is not clear how glucose oxidation is increased by zinc in this cell-based system. Therefore, the aim of this study was to investigate whether zinc increase the expression and translocation of Glut4 to the cell membrane in mouse C2C12 skeletal muscle cells.

This work demonstrated that zinc could increase the expression of Glut4 in a time-dependent manner but could not translocate Glut4 to the cell membrane in C2C12 skeletal muscle cells. Although it is well established that insulin stimulation of glucose uptake in skeletal muscle cells is mediated through translocation of Glut4 from intracellular storage sites to the cell surface (5), according to the results of confocal microscopy, the localisation of Glut4 in skeletal muscle cells was unchanged in cells treated with insulin or zinc when compared to the control cells not treated with these molecules. It is unclear why insulin could not modulate the translocation of Glut4 to the plasma membrane, given that this process is well-established (15). Moreover, the effect of zinc on Glut4 translocation was also negative. Accordingly, some clarification of the experimental design needs to be addressed. This is given in the following paragraphs below.

Previous study identified that zinc enhanced glucose transport independent of insulin in 3T3-L1 fibroblasts (10). Although this study identified that zinc significantly increased tyrosine phosphorylation of the insulin receptor and Akt, it was not identified whether this metal ion could potentiate the mobilisation and translocation of Glut4 in this system. Another study clearly identified that a reduction of the zinc transporter Zip7 in C2C12 skeletal muscle cells, significantly decreased glucose oxidation and this was concomitant with a reduction in Glut4 mRNA and protein levels (16). Again, this study did not test the ability of zinc to translocate Glut4 in the C2C12 skeletal muscle cells. Recent studies identified that zinc treatment in both mouse C2C12 and human skeletal muscle cells significantly contributed to an increase in glucose oxidation and suggested that Glut4 was involved in these processes (7), however, this was not tested. To our knowledge, this is the first result showing zinc can increase Glut4 expression in C2C12 skeletal muscle cells.

Western blot analysis on sub-fractionated cells (membrane versus cytosol) was utilised to determine if Glut4 was mobilised to the cell membrane in skeletal muscle cells. Accordingly, C2C12 cells were treated with insulin or zinc and the cytosolic and membrane fractions were isolated followed by subsequent Western blot with Glut4 antibodies. It was observed that treatment of insulin or zinc localised Glut4 to the membrane fraction of the cells and not the cytosol. However, in the untreated control cells, Glut4 was localised to the membrane and not the cytosol. Glut4 localised in the cytosol is contained within intracellular vesicles that have a plasma membrane (17). Translocation of the Glut4 cytosolic vesicle to the outer plasma membrane occurs through a fusion event that results in the cytosolic vesicle fusing with the plasma membrane to expose Glut4 (18). Thus, the extraction utilised in this experiment sub-fractionates separately the cytosol and membrane components. Given that Glut4 is contained within a membrane-bound vesicle localised in the cytosol, it is highly feasible that this component was extracted in the membrane fraction. This would therefore explain the lack of cytosolic Glut4. Moreover, the control Gapdh used in these experiments suggest this to be the case. Gapdh is a glycolytic enzyme that is solely localised in the cytosol (19). These studies identified abundant expression of Gapdh in the cytosolic fraction as expected and a small amount of expression in the membrane fraction. The expression of Gapdh in the membrane fraction is possibly due to cross-contamination. It has been noted that the kit used in these experiments, Qproteome Cell Extraction Kit (Qiagen), can result in substantial contamination of membrane fractions with cytosolic proteins (20).

The studies also aimed to address the mobilisation and translocation of Glut4 to the plasma membrane in C2C12 skeletal muscle cells following zinc treatment. Initially an antibody directed at the endogenous Glut4 was utilised in the presence of insulin (control) and zinc. The data presented here shows Glut4 expression is confined to the cytosol and no translocation of Glut4 was observed on the outer plasma membrane in both insulin and zinc treated C2C12 cells when compared to the non-treated control cells. It was not clear why insulin-stimulated skeletal muscle cells did not cause a translocation of Glut4 to the outer plasma membrane surface. A previous study suggested that Glut4 is localised intracellularly in C2C12 cells despite insulin stimulation (21). The authors suggest that C2C12 cells exhibit little, if any, insulin-stimulated glucose uptake. However, this is in contrast with data showing a significant increase in glucose uptake in C2C12 cells stimulated with 200 nM insulin (22) and with our previous data showing 10 nM of insulin was sufficient to activate glucose uptake in C2C12 and human skeletal muscle

cells (7). In fact, several studies have reported that C2C12 cells have the basic Glut4 translocation machinery that can be activated by insulin (23-25).

The concentration of insulin and treatment time in this study was 100 nM and 15 minutes, respectively. Other studies have used similar concentrations of insulin but varied the time of the treatment. For example, sixty minutes of insulin treatment at 100 nM was sufficient to activate Glut4 machinery and translocation to the outer plasma membrane (23). Thus, it could be that a greater time of treatment with insulin is required to activate the Glut4 machinery. Unfortunately, there is a lack of data showing zinc-mediated mobilisation and translocation of Glut4 to the outer membrane in skeletal muscle, or any other cell type.

One of the main issues with muscle cultures *in vitro* is the lack of a fully differentiated phenotype, lower expression of Glut4, reduced response to insulin, and an underdeveloped tubular network (26). To overcome this problem often an exogenous system is utilised in the form of a Glut4-GFP labelled plasmid. To this end, similar results were obtained for the transfection of exogenous Glut4 in C2C12 cells where a district intracellular localisation was observed following both insulin and zinc stimulation. Others have previously reported that stably expressing heterologous Glut4 in Chinese hamster ovary (CHO) cells, that insulin-stimulated translocation of the transfected Glut4 was not detectable by immunoblot analysis of subcellular fractions, nor by immunofluorescence microscopy (13, 27). It could be argued that the overexpression of exogenous Glut4 might fail to fold correctly and therefore this would compromise its ability to translocate to the outer plasma membrane. Although this is possible, it is unlikely due to the clear observations of a GFP signal. The GFP signal is important in these studies and will not fluoresce unless the protein of interest (Glut4 in this case) is correctly folded (26).

Similarly, the studies using zinc as a treatment and measuring both the endogenous and exogenous Glut4, resulted in no translocation of Glut4 to the outer plasma membrane (7). While zinc can certainly mediate glucose oxidation in C2C12 cells (7, 16), it is not clear how. Potentially, similar problems exist as outlined above for insulin treatment of C2C12 cells and Glut4 translocation. Alternatively, zinc might require other mechanisms either overlapping, or distinct from the insulin signalling axis to mediate glucose uptake in C2C12 cells. Evidence for this is lacking, although recently we showed that inhibition of the insulin receptor with

HNMPA-(AM)3 resulted in a significant reduction in zinc-mediated pAkt (7). These data suggest that zinc potentially acts through the insulin receptor signalling axis and therefore might explain the lack of response knowing that insulin also was not capable of translocating Glut4 to the plasma membrane. Accordingly, more research is required to delineate the mechanisms of insulin-zinc mediated glucose metabolism in skeletal muscle.

In summary, zinc treatment increases Glut4 protein expression in mouse C2C12 skeletal muscle cells. However, there was no observable changes in insulin or zinc-mediated Glut4 translocation to the plasma membrane in these cells. Additional studies, alongside skeletal muscle cells, using adipocytes which express Glut4 may help delineate the mechanism of zinc-mediated glucose transport.

References

1. Simmons RA. Cell glucose transport and glucose handling during fetal and neonatal development. Fetal and Neonatal Physiology: Elsevier; 2017. p. 428-35. e3.
2. Du K, Murakami S, Sun Y, Kilpatrick CL, Luscher B. DHHC7 palmitoylates glucose transporter 4 (Glut4) and regulates Glut4 membrane translocation. Journal of Biological Chemistry. 2017;292(7):2979-91.
3. Wang T, Li L, Hong W. SNARE proteins in membrane trafficking. Traffic (Copenhagen, Denmark). 2017;18(12):767-75.
4. Ijuin T, Takenawa T. SKIP negatively regulates insulin-induced GLUT4 translocation and membrane ruffle formation. Molecular and cellular biology. 2003;23(4):1209-20.
5. Nedachi T, Kanzaki M. Regulation of glucose transporters by insulin and extracellular glucose in C2C12 myotubes. Am J Physiol-Endocrinol Metab. 2006;291(4):E817-E28.
6. Lauritzen HP, Schertzer JD. Measuring GLUT4 translocation in mature muscle fibers. Am J Physiol-Endocrinol Metab. 2010;299(2):E169-E79.
7. Norouzi S, Adulcikas J, Sohal SS, Myers S. Zinc stimulates glucose oxidation and glycemic control by modulating the insulin signaling pathway in human and mouse skeletal muscle cell lines. PLOS ONE. 2018;13(1):e0191727.
8. Cruz KJC, Morais JBS, de Oliveira ARS, Severo JS, do Nascimento Marreiro D. The Effect of Zinc Supplementation on Insulin Resistance in Obese Subjects: a Systematic Review. Biological Trace Element Research. 2016:1-5.
9. Wu Y, Lu H, Yang H, Li C, Sang Q, Liu X, et al. Zinc stimulates glucose consumption by modulating the insulin signaling pathway in L6 myotubes: essential roles of Akt–GLUT4, GSK3β and mTOR–S6K1. The Journal of nutritional biochemistry. 2016;34:126-35.
10. Tang X-h, Shay NF. Zinc Has an Insulin-Like Effect on Glucose Transport Mediated by Phosphoinositol-3-Kinase and Akt in 3T3-L1 Fibroblasts and Adipocytes. The Journal of Nutrition. 2001;131(5):1414-20.
11. Mahmood T, Yang P-C. Western blot: technique, theory, and trouble shooting. North American journal of medical sciences. 2012;4(9):429.
12. Vaughan R, Gannon N, Barberena M, Garcia-Smith R, Bisoffi M, Mermier C, et al. Characterization of the metabolic effects of irisin on skeletal muscle in vitro. Diabetes, Obesity and Metabolism. 2014;16(8):711-8.
13. Wang Q, Khayat Z, Kishi K, Ebina Y, Klip A. GLUT4 translocation by insulin in intact muscle cells: detection by a fast and quantitative assay. FEBS letters. 1998;427(2):193-7.
14. Samad MB, Mohsin MNAB, Razu BA, Hossain MT, Mahzabeen S, Unnoor N, et al. [6]-Gingerol, from Zingiber officinale, potentiates GLP-1 mediated glucose-stimulated insulin secretion pathway in pancreatic β-cells and increases RAB8/RAB10-regulated membrane presentation of GLUT4 transporters in skeletal muscle to improve hyperglycemia in Lepr db/db type 2 diabetic mice. BMC complementary and alternative medicine. 2017;17(1):395.
15. Kelley DE, Reilly JP, Veneman T, Mandarino L. Effects of insulin on skeletal muscle glucose storage, oxidation, and glycolysis in humans. Am J Physiol-Endocrinol Metab. 1990;258(6):E923-E9.
16. Myers SA, Nield A, Chew GS, Myers MA. The zinc transporter, Slc39a7 (Zip7) is implicated in glycaemic control in skeletal muscle cells. PLoS One. 2013;8(11):e79316.
17. Peppa M, Koliaki C, Nikolopoulos P, Raptis SA. Skeletal Muscle Insulin Resistance in Endocrine Disease. Journal of Biomedicine and Biotechnology. 2010;10.1155/2010/527850.
18. Bevan P. Insulin signalling. Journal of Cell Science. 2001;114(8):1429-30.
19. White MR, Garcin ED. D-Glyceraldehyde-3-Phosphate Dehydrogenase Structure and Function. Sub-cellular biochemistry. 2017;83:413-53.
20. Bünger S, Roblick UJ, Habermann JK. Comparison of five commercial extraction kits for subsequent membrane protein profiling. Cytotechnology. 2009;61(3):153-9.

21. Tortorella LL, Pilch PF. C2C12 myocytes lack an insulin-responsive vesicular compartment despite dexamethasone-induced GLUT4 expression. American journal of physiology Endocrinology and metabolism. 2002;283(3):E514-24.
22. Balasubramanian R, Robaye B, Boeynaems J-M, Jacobson KA. Enhancement of Glucose Uptake in Mouse Skeletal Muscle Cells and Adipocytes by P2Y6 Receptor Agonists. PLOS ONE. 2015;9(12):e116203.
23. Nedachi T, Kanzaki M. Regulation of glucose transporters by insulin and extracellular glucose in C2C12 myotubes. American journal of physiology Endocrinology and metabolism. 2006;291(4):E817-28.
24. Li H, Ou L, Fan J, Xiao M, Kuang C, Liu X, et al. Rab8A regulates insulin-stimulated GLUT4 translocation in C2C12 myoblasts. FEBS Letters. 2017;591(3):491-9.
25. Meng ZX, Gong J, Chen Z, Sun J, Xiao Y, Wang L, et al. Glucose Sensing by Skeletal Myocytes Couples Nutrient Signaling to Systemic Homeostasis. Molecular cell. 2017;66(3):332-44.e4.
26. Lauritzen HP. In vivo imaging of GLUT4 translocation. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme. 2009;34(3):420-3.
27. Shibasaki Y, Asano T, Lin JL, Tsukuda K, Katagiri H, Ishihara H, et al. Two glucose transporter isoforms are sorted differentially and are expressed in distinct cellular compartments. Biochem J. 1992;281 (Pt 3):829-34.

CHAPTER 5

THE ZINC TRANSPORTER ZIP7 IS DOWNREGULATED IN THE SKELETAL MUSCLE OF INSULIN RESISTANT CELLS AND HIGH FAT FED MICE

cells

MDPI

Article

The Zinc Transporter Zip7 Is Downregulated in Skeletal Muscle of Insulin-Resistant Cells and in Mice Fed a High-Fat Diet

Shaghayegh Norouzi [1], John Adulcikas [1], Darren C Henstridge [1,2], Sabrina Sonda [1], Sukhwinder Singh Sohal [1] and Stephen Myers [1,*]

1 College of Health and Medicine, School of Health Sciences, University of Tasmania, Hobart, Tasmania 7005, Australia
2 Baker Heart and Diabetes Institute, Melbourne, Victoria 3004, Australia
* Correspondence: stephen.myers@utas.edu.au; Tel.: +61-3-6324-5459

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Abstract: Background: The zinc transporter Zip7 modulates zinc flux and controls cell signaling molecules associated with glucose metabolism in skeletal muscle. The present study evaluated the role of Zip7 in cell signaling pathways involved in insulin-resistant skeletal muscle and mice fed a high-fat diet. Methods: Insulin-resistant skeletal muscle cells were prepared by treatment with an inhibitor of the insulin receptor, HNMPA-(AM)3 or palmitate, and Zip7 was analyzed along with pAkt, pTyrosine and Glut4. Similarly, mice fed normal chow (NC) or a high-fat diet (HFD) were also analyzed for protein expression of Glut4 and Zip7. An overexpression system for Zip7 was utilized to determine the action of this zinc transporter on several genes implicated in insulin signaling and glucose control. Results: We identified that Zip7 is upregulated by glucose in normal skeletal muscle cells and downregulated in insulin-resistant skeletal muscle. We also observed (as expected) a decrease in pAkt and Glut4 in the insulin-resistant skeletal muscle cells. The overexpression of Zip7 in skeletal muscle cells led to the modulation of key genes involved in the insulin signaling axis and glucose metabolism including *Akt3*, *Dok2*, *Fos*, *Hras*, *Kras*, *Nos2*, *Pck2*, and *Pparg*. In an *in vivo* mouse model, we identified a reduction in Glut4 and Zip7 in the skeletal muscle of mice fed a HFD compared to NC controls. Conclusions: These data suggest that Zip7 plays a role in skeletal muscle insulin signaling and is downregulated in an insulin-resistant, and HFD state. Understanding the molecular mechanisms of Zip7 action will provide novel opportunities to target this transporter therapeutically for the treatment of insulin resistance and type 2 diabetes.

Keywords: zinc; zinc transporter; Zip7; insulin signaling pathway; high-fat diet; skeletal muscle

1. Introduction

It was estimated in 2013 that 382 million people were affected by diabetes and this number is expected to increase to 592 million by 2035 [1]. This rapid escalation in diabetes can be attributed to rapid economic developments and lifestyle changes associated with reduced physical activity and an increase in the consumption of high calorie diets, resulting in a higher obesity prevalence [2]. Adipose tissue dysfunction is one mechanism responsible for systemic metabolic complications, such as type-2 diabetes (T2D) [3,4]. T2D is characterized by insulin resistance in major peripheral metabolic tissues including adipose tissue, liver and skeletal muscle. While most obese individuals do not become diabetic even in the face of a high degree of insulin resistance, most patients with T2D are obese [5]. Thus, in addition to insulin resistance, compromised pancreatic β-cell function leads to increased blood glucose and contributes to hyperglycemia and overt T2D [6].

Research on T2D has revealed a role for the physiological importance of zinc and the proteins that transport this metal ion in cells (zinc transporters) in diseases associated with abnormal cell signaling pathways and metabolism such as insulin resistance (IR) and T2D [7]. Zinc is a vital trace element that is present in organs, tissues, fluids and secretions and functions as a critical cofactor in an extensive number of biological signaling pathways as a catalytic, structural or regulatory component [8]. The storage, release and distribution of cellular zinc are regulated by a family of zinc transporter proteins and metallothioneins. In mammals, there are 24 zinc transporter proteins divided into two families. These are the zinc efflux (Slc30/ZnT, members 1–10) and the zinc influx (Slc39/ZIP, members 1–14) proteins [9]. The ZnT members function to transport zinc from the cell or into subcellular organelles when cytoplasmic zinc is high. In contrast, the ZIP members function to transport zinc into the cell when zinc in the cytosol is low or depleted [10].

Emerging research has highlighted key roles for zinc transporter ZIP7 in signaling pathways. ZIP7 is located in the endoplasmic reticulum (ER) membrane and it is regulated in response to phosphorylation by casein kinase II (CK2), an enzyme that promotes cell division [11]. ZIP7 is a gatekeeper of cytosolic zinc release from subcellular organelles including the ER and Golgi apparatus [12]. It has been demonstrated that genetic ablation of *ZIP7* resulted in reduced cytosolic zinc levels, and abnormalities in cell proliferation and ER function in human osteosarcoma cell lines [13]. Similarly, dysfunctional ZIP7 caused proliferation of the tamoxifen-resistant MCF-7 breast cancer phenotype [14]. Recent data on zinc transporters also suggests that Zip7 is implicated in glucose metabolism and glycemic control in skeletal muscle cells [15]. The ablation of *Zip7* in skeletal muscle cells resulted in a substantial reduction in several genes and proteins involved in glucose homeostasis. These included the phosphorylation of Akt, the insulin receptor (Ir), insulin receptor substrates 1 and 2 (Irs1 and Irs2), the glucose transporter Glut4, and glycogen branching enzyme (Gbe). Similarly, studies identified a redistribution of cellular ER zinc in hyperglycemic rat heart cells that involved changes in Zip7 protein and Zip7 phosphorylation [16]. Given the role of Zip7 in regulating zinc flux and the activation of key cell signaling molecules associated with glucose metabolism, we propose that this transporter controls cell signaling pathways involved in glucose metabolism in skeletal muscle.

2. Materials and Methods

2.1. Cell Culture

Mouse C2C12 cells were obtained from Professor Steve Rattigan, Menzies Institute for Medical Research, Hobart, Australia. C2C12 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher, Victoria, Australia) medium that contained 10% fetal calf serum (FCS) and 100 U/mL penicillin/streptomycin (Thermo Fisher) and were maintained at 37 °C and 5% CO_2 in a humidified atmosphere. C2C12 cells were differentiated into myotubes by the addition of media containing 2% horse serum (Thermo Fisher) for seventy-two hours. The cells were then exposed to serum-free conditions for three hours prior to the different treatments as outlined below.

2.2. Protein Extraction

Whole cell protein lysates were prepared in RIPA Lysis buffer in the presence of protease and protein phosphatase inhibitors (Thermo Fisher) as previously described [17]. Briefly, whole cell lysates were vortexed every 10 min for 1 h on ice and centrifuged at 15,000 rpm for 5 min. The protein concentrations of the supernatants were determined by a BCA assay kit as per manufacturer's instructions (Thermo Fisher).

2.3. RNA Extraction

Total RNA was extracted using the Qiagen RNeasy Mini Kit as per manufacturer's instructions (Qiagen, Victoria, Australia). Briefly, cells were lysed in RLT Buffer, placed directly into a QIAshedder spin column and centrifuged for 2 min. Lysates were then passed through a RNeasy spin column and

purified by adding RW1 and RPE buffer. The purified RNA was eluted in RNAse-free water and total RNA concentration was determined by UV spectrometry.

2.4. cDNA Synthesis

Complementary DNA (cDNA) was synthesized from extracted total RNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Victoria, Australia) and using random hexamers according to the manufacturer's instructions. Briefly, 10 µL cDNA reverse-transcription mix was added to 10 µL genomic DNA elimination mix and incubated at 42 °C for 15 min. The reaction was stopped by incubating the samples at 95 °C for 5 min. The resulting reverse transcription products were stored at −20 °C until use.

2.5. Mice and Diets

The experimental procedures for all animal work has been previously described and the mice used in these experiments represent a sub group of a previously published cohort of animals [18]. All experiments involving the use of animals for research were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and were conducted in accordance with the National Health and Medical Research Council of Australia guidelines. Ethics number E/1255/2012/B. Mice (six mice per group) were fed either a normal chow (NC) diet (14.3 MJ/kg, 76% of kJ from carbohydrate, 5% fat, 19% protein) or a high-fat diet (HFD), (19 MJ/kg, 36% of kJ from carbohydrate, 43% fat [42.7% saturated, 35.1% monounsaturated and 21.7% polyunsaturated fatty acids] and, 21% protein), Specialty Feeds, Glen Forrest, Western Australia, Australia). During the experiment, the animals were given their prescribed diet and water ad libitum, except for fasting periods before a glucose tolerance test and were housed at 22 °C on a 12-h light/dark cycle.

2.6. Body Composition Analysis

Body mass was measured using standard laboratory scales (Mettler Toledo, Greifensee, Switzerland). Lean and fat mass was measured using a 4-in-1 EchoMRI (Houston, TX, USA).

2.7. Oral Glucose Tolerance Test

Mice were fasted for 6 h and subsequently received an oral gavage of 2 g glucose/kg lean body mass (25% *w/v* glucose solution). An oral glucose tolerance test (OGTT) was performed and glucose levels were measured from tail tip blood at 0, 15, 30, 45, 60, 90 and 120 mins.

2.8. Mouse Tissue Collection and Protein Extraction

Mice were sacrificed by sodium pentobarbital overdose. Skeletal muscle quadriceps were used for analysis. Skeletal muscles used for protein analysis were frozen on dry ice and stored at −80 °C until analysis. A protease inhibitor cocktail was added to the CelLytic MT (Mammalian Tissue Lysis/Extraction Reagent, Sigma) reagent and a ratio of tissue to CelLytic MT reagent of 1:20 was used for tissue protein extraction. Samples were then transferred to a pre-chilled microhomogenizer and tissues were homogenized. The lysed samples were centrifuged for 10 min at 12,000–20,000× *g* to pellet the tissue debris. The protein- containing supernatants were transferred to a chilled test tube and stored at −80 °C. Protein concentrations of the supernatants were determined using the BCA assay kit as per manufacturer's instructions (Thermo Fisher).

2.9. Fatty Acid Preparation and Cell Culture Treatment

Palmitate was prepared as previously described [19]. Briefly, fatty acid was mixed with ethanol to a final concentration of 100 mM. Then the mixture was sonicated on ice at 200W with 10-sec bursts, 3-sec off pulses until the mixture became a milky homogenous solution. Prepared fatty acid stock was kept at 4 °C and protected from light. For fatty acid treatment, the fatty acid stock solution was

added to the cell culture medium containing 2% horse serum at 60 °C to a final concentration as stated. For the control, the same amount of ethanol was added to the cell culture medium containing 2% horse serum. Cells were cultured with increasing concentrations of palmitate-containing media for 24 h followed by 10 nM insulin treatment for 30 min prior to cell lysate harvest. Following treatment, whole cell lysates were prepared and immunoblot analysis was performed on pAkt, Zip7, glucose transporter 4 (Glut4), phospho-tyrosine and Gapdh.

2.10. Cell Viability Assay [1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan] Assay

Cell viability was measured using the MTT (3-(4,5)-dimethylthiahiazo(−z-y1)-3,5-diphenytetrazoliumromid) assay as per manufacturer's instructions (Thermo Fisher). Briefly, C2C12 skeletal muscle cells were seeded at a density of 2×10^4 cells/well in a 96-well plate. Following C2C12 differentiation the cells were treated with varying concentrations of palmitate (as described in the results) for 24 h. Then 15 µL of 5 mg/mL MTT was added to each well. Following a 4 h incubation at 37 °C, the produced formazan was solubilized in 150 µL dimethyl sulfoxide (DMSO). The absorbance was measured at 490 nm using a microplate reader (TECAN infinite M200 PRO, Männedorf, Switzerland).

2.11. Insulin Receptor Inhibition

Insulin receptor tyrosine kinase activity is inhibited by 50 µM insulin receptor tyrosine kinase inhibitor hydroxy-2-naphthalenylmethylphosphonic acid trisacetoxymethyl ester (HNMPA-(AM)3) after 1 h. It suppresses the activation of pAkt after 30 min of insulin treatment [17]. C2C12 skeletal muscle cells were treated with the insulin receptor inhibitor [HNMPA-(AM)3] (Abcam, Victoria, Australia) for 1 h at 0, 25 and 50 µM. This was followed by treating the C2C12 cells with 10 nM insulin for 30 min. Following treatment, whole cell protein lysates were extracted and immunoblot analysis was performed on pAkt, Zip7, glucose transporter 4 (Glut4) and Gapdh.

2.12. Western Blotting

Tissue or cell-extracted proteins (20 µg) were electrophoresed using 4–15% SDS-polyacrylamide gels (Bio-Rad, New South Wales, Australia) and immunoblotted using semi-dry electrophoresis blotting system. Phosphorylated Akt (1:5000), Zip7 (1:1000) or Glut4 (1:1000) (Cell Signaling Technology, Beverly, MA, USA) primary antibodies were used for immunoblotting. Antibodies were detected with HRP-linked secondary antibodies (1:5000) (Cell Signaling Technology, USA). Then, membranes were stripped with Restore Plus Western Blot Stripping Buffer (Thermo Fisher) and reprobed for the related housekeeping gene (Gapdh 1:2000 or total Akt 1:5000, Cell Signaling Technology, USA). Proteins were visualized with SuperSignal West Femto Kit (Thermo Fisher) and imaged using an Odyssey infrared imaging system (LiCor, Millennium Science, Victoria, Australia). Relative band density was quantified using Image J software. Representative immunoblots are provided. Experiments were repeated three times and statistical analysis was performed on the signal intensity of the protein of interest relative to the house keeping gene on all protein bands from each of the experiments.

2.13. Zip7 Overexpression

To determine the role of Zip7 in modulating cellular pathways, a plasmid-based system was constructed that overexpressed the Zip7 protein (Origene, Rockville, MD, USA, Clone ID MR216531; plasmid cytomegalovirus, pCMV-Zip7). The vector was transformed into high-efficiency bacterial cells and plasmid DNA was isolated using an ISOLATE Plasmid Kit. The bacterial transformation was performed by heat-shock at 42 °C for 30 s as described by the manufacturer's instructions (Thermo Fisher). The transformed bacteria were plated onto LB agar plates containing appropriate antibiotics and incubated overnight at 37 °C. Bacterial colonies were harvested and plasmids isolated using an ISOLATE II plasmid midi kit as per the manufacturer's instructions (Bioline, New South Wales, Australia). Restriction digest analysis using the enzymes Sgf1 and and Mlu1 was used to determine

the correct insertion of the *Zip7* gene from the plasmid. *Zip7* containing plasmids were transfected into skeletal muscle cells using Lipofectamine 3000 reagent (Thermo Fisher) as per manufacturer's instructions. Briefly, skeletal muscle cells were cultured as previously described in 6-well dishes. Once the cells had reached 70% confluence, approximately 1 μg of pCMV-*Zip7* (and pCMV empty vector) was mixed with the Lipofectamine 3000 in DMEM and placed on the cells. After 24 h the cells were harvested for RNA and protein. The quality and quantity of RNA were assessed by nucleic acid spectrometry (Bio-Rad) and the measurement of optical density at 260 and 280 nm.

2.14. Quantitative Real-Time PCR (qPCR)

To determine whether Zip7 is overexpressed in skeletal muscle cells, qPCR was performed as previously described [15]. qPCR was performed using 20 ng of cDNA template with 2 x Sensi Fast Sybr (Bioline) as per manufacturer's instructions. The relative level of target gene expression was normalized to eukaryotic elongation factor 2 (Eef2). Primers to amplify the Zip7 sequence were designed using PrimerFind (NCBI: https://www.ncbi.nlm.nih.gov). Sequences of the primers were as follows: Zip7 (forward: 5′- CGC ATG CCT TGG AAC CTC AT- 3′; Reverse: 5′- GGC GAC AAT CCC ACT GAG AA-3′), Eef2 (Forward: 5′- CAC AAT CAA ATC CAC CGC CAT-3′; Reverse: 5′- TGG CCT GGA GAG TCG ATG A- 3′) (Integrated DNA Technologies, Singapore Science Park II, Singapore). RT^2 qPCR Primer Assay for Mouse Slc2a4, (Qiagen), was used to amplify the Glut4 sequence.

2.15. Insulin Signaling Pathway Array

Insulin Signaling Pathway Array (SABiosciences, Qiagen) was utilized to detect the expression of 84 genes implicated in the insulin signaling pathway. cDNA from the control (empty vector; pCMV) and pCMV-*Zip7* transfected C2C12 cells was assayed across the array. Real-time PCR was performed to analyze the expression of a focused panel of genes related to insulin-responsiveness and results were analyzed using GeneGlobe Data Analysis Center (Qiagen, Australia).

2.16. Data Analysis

Results are presented as means ± SDs. Statistical comparisons were performed using Student's *t*-test (Prism GraphPad, version 8, San Diego, CA, USA) for mRNA expression and protein analysis. A significant effect was demonstrated at $p < 0.05$. For mouse studies, differences between normal chow (NC) and high-fat diet (HFD) (diet effect) were analyzed by a paired *t*-test. For the OGGT, two-way analysis of variance (ANOVA) with a *post-hoc* analysis (Bonferroni) was used to detect the effect of diet on glucose clearance. Analysis was performed by Prism GraphPad (version 8). A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Glucose Regulates Zip7 Expression in C2C12 Skeletal Muscle Cells

Previously we have shown that reduced *Zip7* in C2C12 skeletal muscle cells led to a significant decrease in the phosphorylation of Akt, reduced Glut4 expression and compromised insulin-mediated glycogen synthesis [15]. Accordingly, we wanted to test whether glucose could regulate Zip7 in C2C12 skeletal muscle cells to establish a role for this transporter in glycemic control. Previous studies have reported that Zip7 is upregulated by glucose in rat cardiomyocytes [16] and mouse pancreatic islets [20]. Accordingly, treatment of C2C12 cells with 0, 10 and 25 mM glucose for 2 h resulted in a robust increase in the protein expression of Zip7 (Figure 1a,b).

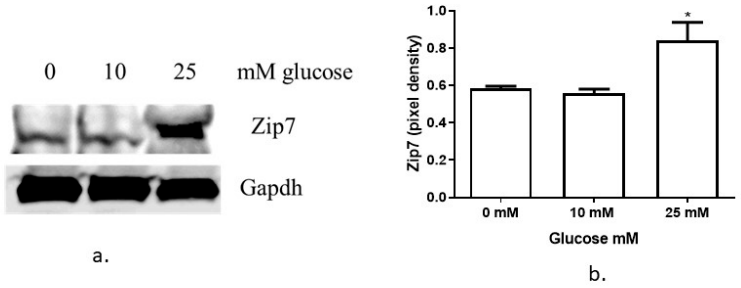

Figure 1. Analysis of Zip7 in mouse skeletal muscle cells treated with glucose. (**a**) Increasing concentrations of glucose (0, 10 and 25 mM) were added to C2C12 cells over 24 h. Gapdh was used as an internal loading control and levels of Zip7 were normalized to Gapdh. Representative image of three independent western blot assays that were performed on three independent treatments ($n = 3$). (**b**) Densitometry graphs for Zip7 from three independent data westerns blots. * = $p < 0.05$.

3.2. The Expression of Zip7 and Glut4 Is Suppressed in Insulin-Resistant C2C12 Cells Treated with Either an Insulin Receptor Inhibitor Hnmpa-(Am)3 or Palmitate

Given that Zip7 could be regulated by glucose in C2C12 skeletal muscle cells, we decided to perform several studies to recapitulate a compromised glucose state in skeletal muscle by creating insulin-resistant C2C12 cells. To recapitulate an insulin-resistant state in skeletal muscle we utilized two experimental procedures: (1) an inhibitor of the insulin receptor and, (2) palmitate treatment [19,21,22]. Initially we tested the ability of the insulin receptor inhibitor HNMPA-(AM)3 to inhibit the insulin signaling pathway by measuring the downstream target of the insulin receptor, Akt. It has been previously shown that 50 µM of HNMPA-(AM)3 is sufficient to inhibit insulin-induced pAkt in C2C12 cells [19]. Accordingly, 50 µM of HNMPA-(AM)3 was sufficient to inhibit the insulin-dependent activation of pAkt in C2C12 skeletal muscle cells (Figure 2a,b). We also assayed the levels of Zip7 and Glut4 in the HNMPA-(AM)3 treated C2C12 cells and identified a significant reduction in the levels of these proteins (Figure 2a,c,d).

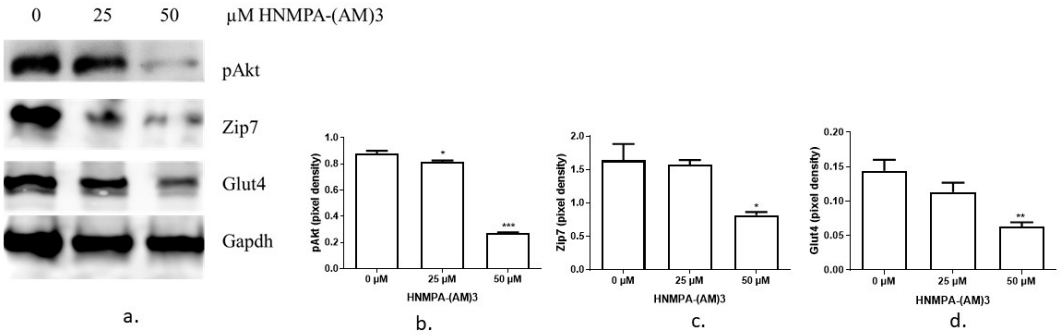

Figure 2. Western blot results of pAkt, Zip7 and Glut4 expression in C2C12 cells treated with insulin receptor tyrosine kinase inhibitor HNMPA-(AM)3. (**a**) The inhibitor concentration was 0, 25 and 50 µM. From the top the proteins are, pAkt, Zip7, Glut4 and Gapdh. Gapdh was used as an internal loading control. Three independent western blots on three independent treatments were performed (n = 3). (**b**–**d**). Densitometry graphs for pAkt, Zip7 and Glut4, respectively from three independent data westerns blots. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

It has been shown previously that saturated fatty acid palmitate reduces insulin sensitivity and induces insulin resistance in skeletal muscle [18]. In this experiment, the inhibitory effect of palmitate on the insulin signaling pathway in C2C12 myotubes was tested. Initially we aimed to test the viability of the C2C12 cells following palmitate treatment. The result of the MTT assay showed that 0.3 mM of palmitate did not significantly suppress C2C12 cell viability (Figure 3a), while at the same time,

it suppressed insulin-stimulated phosphorylation of Akt (Figure 3b,c). Accordingly, we chose 0.3 mM of palmate as the highest concentration of this fatty acid in the following experiments.

To determine the effect of palmitate treatment on Zip7 we assayed the level of Zip7 protein in palmitate-treated C2C12 cells. The expression level of Zip7 protein were reduced when treated with increasing concentrations of palmitate (Figure 3b,d). We also observed a reduction in pAkt in the presence of palmitate as previously described [19]. Glut4 was also reduced in the presence of increasing palmitate concentrations (Figure 3b,e). We also tested the ability of palmitate to reduce the levels of phospho-tyrosine, a key amino acid involved in cellular signal transduction. Following palmitate treatment, the levels of phospho-tyrosine were also reduced (Figure 3b,f). These results indicate that palmitate treatment was sufficient to induce an insulin-resistant state in C2C12 skeletal muscle cells (as evidence by the reduction in pAkt and phospho-tyrosine).

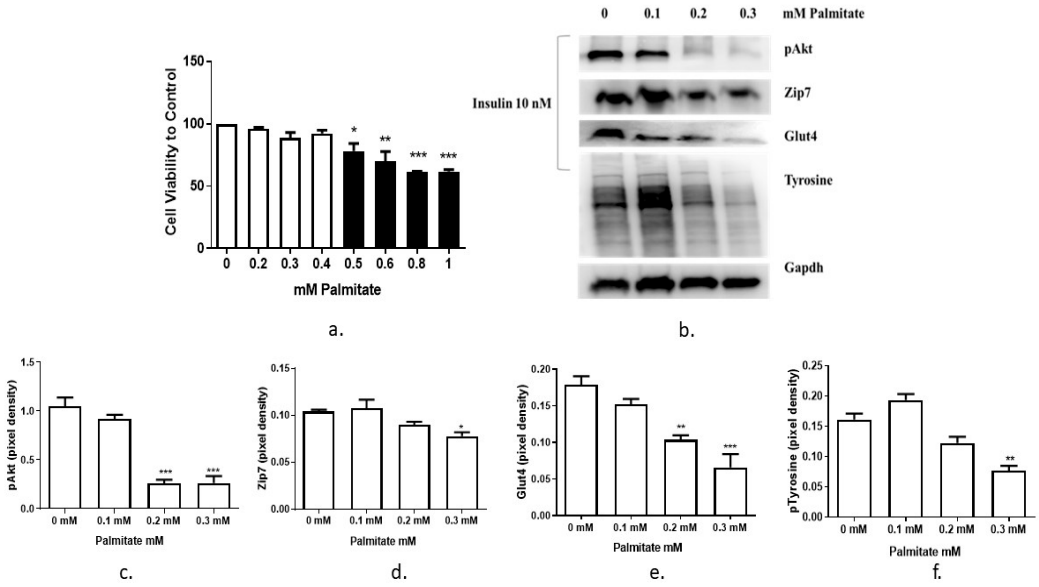

Figure 3. Recapitulation of an insulin-resistant state with palmitate. (**a**) MTT assay for cell viability assay in C2C12 cells treated with increasing concentrations of palmitate. Concentrations of palmitate are 0, 0.2, 0.3 and 0.4 mM (white bars); 0.5, 0.6. 0.8 and 1 mM (black bars). Cell viability was performed three times ($n = 3$). (**b**) Palmitate-induced insulin resistance in C2C12 cells. Cells were treated with 0, 0.1, 0.2 and 0.3 mM palmitate over 24 h, then stimulated with 10 nM insulin for 30 min before harvesting total protein. Phosphorylated Akt (Ser473), Zip7, Glut4 and phosphor-tyrosine were immunoprobed by western blotting. Gapdh was used as an internal control. Three independent western blots on three independent treatments were performed ($n = 3$). (**c**–**f**). Densitometry graphs for pAkt, Zip7, Glut4, and pTyrosine, respectively from three independent data westerns blots * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.3. Testing the Function of ZIP7 in Controlling Genes Involved in Insulin Signaling

The above studies suggest that Zip7 is involved in metabolic processes that could enhance cell signaling pathways associated with glucose metabolism. Accordingly, we wanted to test whether Zip7 could activate genes implicated in insulin signaling and glucose homeostasis. Initially we overexpressed Zip7 in C2C12 skeletal muscle cells utilizing an overexpression plasmid pCMV (Figure 4). We observed a significant increase in pCMV-*Zip7* mRNA compared to pCMV control (Figure 4a). Similarly, we observed a significant increase in pCMV-Zip7 protein compared to pCMV control (Figure 4b,c).

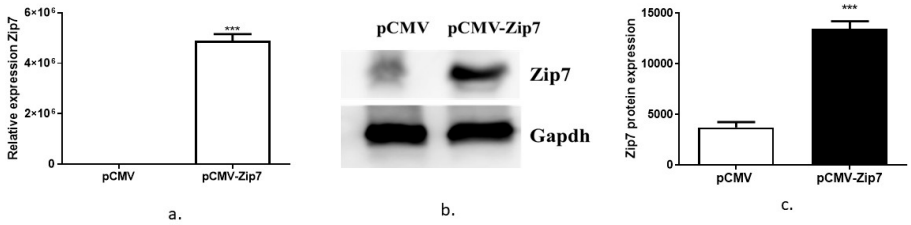

Figure 4. Overexpression of Zip7 in C2C12 skeletal muscle cells. (**a**) Quantitative real-time polymerase chain reaction (PCR) for the overexpression of Zip7 mRNA in pCMV versus pCMV-Zip7. (**b**) Western blot for pCMV-Zip7 protein. Gapdh was used as a loading control. (**c**) Densitometry results from the western blot data pCMV versus pCMV-Zip7. The experiments were performed three times and statistical significance compared ZIP-7 over-expressed group to the control. *** = $p < 0.001$.

To further identify the effect of pCMV-Zip7 overexpression on insulin signaling, we used an insulin signaling pathway array, which has the targeted expression of eighty-four genes involved in insulin signaling and glucose metabolism. The genes were selected based on a commercially available insulin signaling array (SABiosciences, Qiagen) and focused cellular pathways. We tested the pCMV-Zip7 overexpression plasmid to target the expression of genes on this array. Following transfection of the pCMV-Zip7 overexpression plasmid and control empty vector (pCMV) in C2C12 cells, total RNA was extracted, and cDNA synthesis performed. Changes in the expression of all genes on the array following pCMV-Zip7 overexpression transfection are presented as (1) the gene name, (2) fold up or down regulation, and (3) the *p*-value (Table 1). Although we identified several genes with fold-change expression greater than the statistically significant highlighted genes in Table 1 below (in bold), these did not reach significance and did not meet the stringent data normalization processes (GeneGlobe Data Analysis Center Qiagen).

Table 1. Insulin signaling gene array.

Gene Name	Fold–Up or Downregulation	*p*–value
Insulin Receptor–Associated Proteins		
Eukaryotic translation initiation factor 4E binding protein 1 (Eif4ebp1)	−5.42	0.109812
Insulin−like growth factor 2 (Igf2)	−2.51	0.119985
Growth factor receptor bound protein 2 (Grb2)	**−1.81**	**0.044301**
Fibroblast growth factor receptor substrate 3 (Frs3)	−1.78	0.342511
Sorbin and SH3 domain containing 1 (Sorbs1)	−1.57	0.261979
Protein tyrosine phosphatase, receptor type, F (Ptprf)	−1.49	0.376424
Growth factor receptor bound protein 2−associated protein 1 (Gab1)	−1.48	0.237770
Non−catalytic region of tyrosine kinase adaptor protein 1 (Nck1)	−1.24	0.479138
Fibroblast growth factor receptor substrate 2 (Frs2)	−1.21	0.514579
Protein tyrosine phosphatase, non−receptor type 1 (Ptpn1)	−1.19	0.481621
Heat shock protein 90 alpha (cytosolic), class B member 1 (Hsp90ab1)	1.04	0.965684
src homology 2 domain−containing transforming protein C1 (Shc1)	1.13	0.659820
Protein phosphatase 1 catalytic subunit alpha (Ppp1ca)	1.15	0.595936
Casitas B−lineage lymphoma (Cb1)	1.15	0.759472
Insulin receptor substrate 1 (Irs1)	1.47	0.435010
Insulin−like growth factor I receptor (Igfr)	1.54	0.241275
Insulin receptor substrate 2 (Irs2)	1.67	0.240410
Prolactin (Prl)	1.82	0.518438
Insulin 1 (Ins1)	2.04	0.401028
Insulin−like growth factor binding protein 1 (Igfbp1)	2.14	0.391212

Table 1. *Cont.*

Gene Name	Fold–Up or Downregulation	*p*–value
Neuropeptide Y (Npy)	2.60	0.057738
Docking protein 1 (Dok1)	2.91	0.103312
Docking protein 3 (Dok3)	3.30	0.315765
Growth factor receptor bound protein 10 (Grb10)	3.75	0.140065
Insulin−like 3 (InsI3)	4.33	0.287920
Thyroglobulin (Tg)	5.53	0.179122
Docking protein 2 (Dok2)	**21.79**	**0.031048**
PI3 Kinase Signaling		
Thymoma viral proto−oncogene 3 (Akt3)	**−2.16**	**0.019032**
Phosphoinositide−3−kinase regulatory subunit 1 (Pik3r1)	−1.57	0.350159
Thymoma viral proto−oncogene 2 (Akt2)	−1.46	0.384953
Protein kinase C, gamma (Prkcg)	−1.42	0.281971
Eukaryotic translation initiation factor 2B, subunit 1 (alpha) (Eif2b1)	−1.39	0.219592
Phosphatidylinositol−4,5−bisphosphate 3−kinase catalytic subunit alpha (Pik3ca)	−1.38	0.333187
Phosphatidylinositol−4,5−bisphosphate 3−kinase catalytic subunit beta (Pik3cb)	−1.30	0.444354
3−phosphoinositide dependent protein kinase 1 (Pdpk1)	−1.15	0.751995
Protein kinase C, iota (Prkci)	−1.14	0.707935
Mechanistic target of rapamycin kinase (Mtor)	1.08	0.874784
BCL2−like 1 (Bcl2l1)	1.21	0.631682
Phosphoinositide−3−kinase regulatory subunit 2 (Pik3r2)	1.22	0.653162
Adrenergic receptor, alpha 1d (Adra1d)	1.25	0.391856
Thymoma viral proto−oncogene 1 (Akt1)	1.36	0.241863
Dual specificity phosphatase 14 (Dusp14)	1.42	0.465154
Protein kinase C, zeta (Prkcz)	2.00	0.327070
Serine peptidase inhibitor, clade E, member 1 (Serpine 1)	2.09	0.131939
MAP Kinase Signaling		
Related RAS viral (r−ras) oncogene 2 (Rras2)	−1.86	0.075688
Braf transforming gene (Braf)	−1.78	0.127855
Related RAS viral (r−ras) oncogene (Rras)	−1.63	0.070002
Araf proto−oncogene, serine/threonine kinase (Araf)	−1.40	0.331800
SOS Ras/Rac guanine nucleotide exchange factor 1 (Sos1)	−1.21	0.477393
Excision repair cross−complementing rodent repair deficiency, complementation group 1 (Ercc1)	−1.02	0.884782
v−raf−leukemia viral oncogene 1 (raf1)	1.03	0.834133
Uncoupling protein 1 (mitochondrial, proton carrier) (Ucp1)	1.26	0.433977
Mitogen−activated protein kinase kinase 1 (Map2k1)	1.43	0.244012
Mitogen−activated protein kinase 1 (Mapk1)	1.82	0.134200
Kruppel−like factor 10 (Klf10)	2.12	0.143675
Nitric oxide synthase 2, inducible (Nos2)	**2.21**	**0.030364**
Harvey rat sarcoma virus oncogene (Hras)	**2.30**	**0.027193**
Ribosomal protein S6 kinase polypeptide 1 (Rps6ka1)	2.93	0.286011
FBJ osteosarcoma oncogene (Fos)	**4.42**	**0.014490**
Carbohydrate Metabolism		
Phosphoenolpyruvate carboxykinase 2 (mitochondrial) (Pck2)	**−2.73**	**0.046319**
Glucuronidase, beta (Gusb)	**−2.44**	**0.057494**

Table 1. *Cont.*

Gene Name	Fold–Up or Downregulation	*p*–value
Pyruvate kinase liver (Pkl)	−2.00	0.361565
AE binding protein 1 (Aebp1)	−1.36	0.346200
acyl−Coenzyme A oxidase 1, palmitoyl (Acox1)	−1.23	0.517860
Hexokinase 2 (Hk2)	1.06	0.892955
Glycogen synthase kinase 3 beta (Gsk3b)	1.04	0.908617
Glyceraldehyde−3−phosphate dehydrogenase (Gapdh)	1.15	0.642638
Glucose−6−phosphatase, catalytic (G6pc)	1.22	0.432012
Glycerol−3−phosphate dehydrogenase 1 (Gpd1)	1.43	0.434684
Solute carrier family 2 (facilitated glucose transporter), member 1 (Slc2a1)	1.50	0.246541
Glucokinase (Gck)	1.96	0.316235
Fructose bisphosphatase 1 (Fbp1)	3.40	0.315244
Glucose−6−phosphatase, catalytic, 2 (G6pc2)	4.71	0.189288
Lipid Metabolism		
Complement factor D (adipsin) (Cfd)	−3.66	0.370569
Peroxisome proliferator activated receptor gamma (Pparg)	**−3.31**	**0.007128**
Sterol regulatory element binding transcription factor 1 (Srebf1)	**−1.59**	**0.041612**
Resistin (Retn)	−1.71	0.786857
Low density lipoprotein receptor (Ldlr)	−1.11	0.938288
Leptin (Lep)	1.31	0.490170
Solute carrier family 27 (fatty acid transporter), member 4 (Slc27a4)	1.33	0.350357
Cell Growth and Differentiation		
Kirsten rat sarcoma viral oncogene homolog (Kras)	**2.12**	**0.025606**
CCAAT/enhancer binding protein (C/EBP), alpha (Cebpa)	−1.41	0.700291
Vascular endothelial growth factor A (Vegfa)	−1.14	0.660055
CAP, adenylate cyclase−associated protein 1 (Cap1)	−1.13	0.673316
Jun proto−oncogene (Jun)	1.02	0.996474
Beta−2 microglobulin (B2m)	1.13	0.700177
Actin, beta (Actb)	1.28	0.577259
CCAAT/enhancer binding protein (C/EBP), beta (Cebpb)	2.33	0.145381

Note: Genes are listed by specific pathways: insulin receptor and associated proteins, PI3 kinase signaling, MAP kinase signaling, carbohydrate metabolism, lipid metabolism, and cell growth and differentiation. Please also note that several genes can belong to multiple pathways. The fold change for each gene within the pathway is shown initially as the highest value for the downregulation (a negative symbol) followed by a sequential increase in values into the positive range.

Representative graphs are given for the genes that were significantly changed due to the over expression of pCMV-Zip7. These include Akt3, Dok2, Fos, Hras, Kras, Nos2, Pck2, and Pparg, (Figure 5a–h).

3.4. The Expression of Zip7 and Glut4 Is Reduced in the Skeletal Muscle of Mice Fed a High-Fat Diet

The results above suggest that Zip7 has a role in modulating genes involved in glucose metabolism and is reduced in an insulin-resistant state when treated pharmacologically or with fatty acids. This suggests that Zip7 could be regulated by changes in fatty acids or a high-fat diet, for example. To determine if Zip7 is modulated by a high-fat diet we performed experiments on C57BL/6J mice consuming a normal chow (NC) or high-fat diet (HFD) for 10 weeks. As expected on the HFD, body weight, fat mass and body fat percentages were increased compared to the NC-fed mice while lean mass remained unchanged (Figure 6a–d). Next, we performed an oral glucose tolerance test (OGTT) to assess insulin-stimulated glucose clearance. While blood glucose decreased over a 120-min time

course in both NC and HFD cohorts, there was an overall dietary effect on glucose clearance in the HFD mice versus the NC control (Figure 6e).

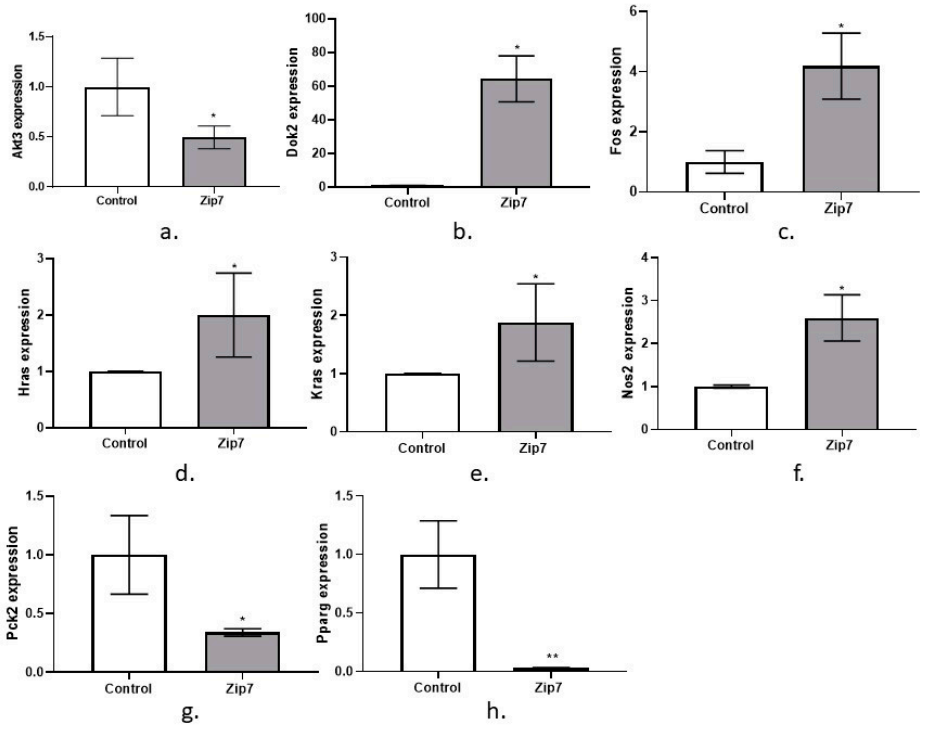

Figure 5. Quantitative real time PCR on an insulin signaling RT-Profiler Array for pCMV-Zip7 overexpression. Genes are (**a**) Akt3, (**b**) Dok2, (**c**) Fos, (**d**) Hras, (**e**) Kras, (**f**) Nos2, (**g**) Pck2, (**h**) Pparg. Control = pCMV empty vector. Zip7 = overexpression pCMV-Zip7. * $p < 0.05$, ** $p < 0.01$.

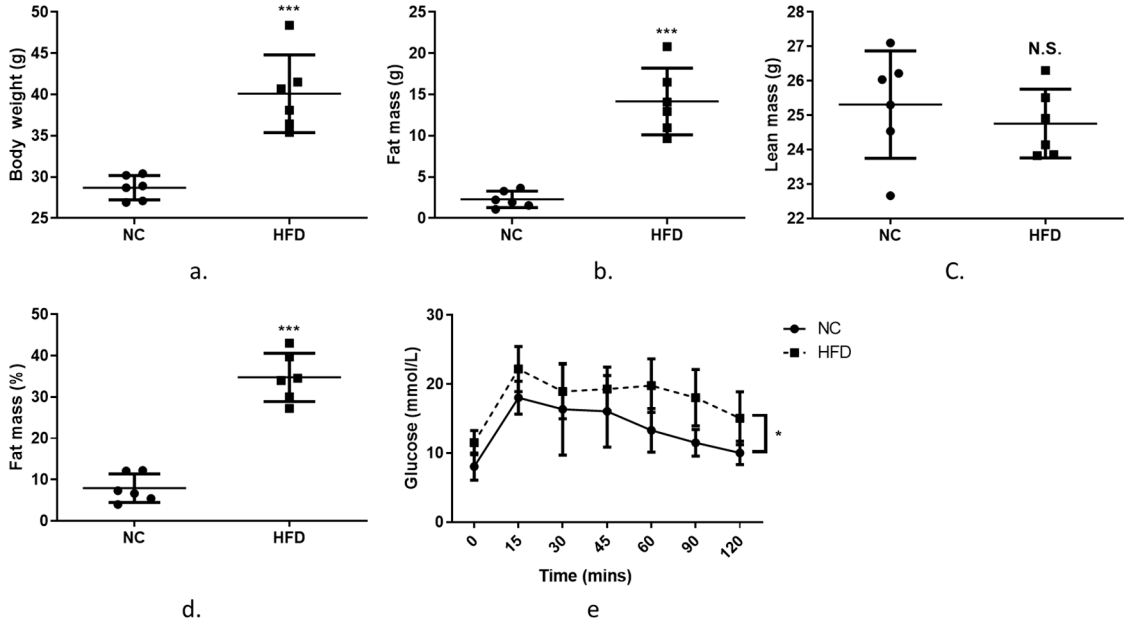

Figure 6. Characteristics of mice fed a normal chow (NC) diet or a high-fat diet (HFD) for 10 weeks. (**a**) body weight (g), (**b**) fat mass (g), (**c**) lean mass (g), (**e**) fat mass (%), (f) plasma glucose levels over 120 min following an oral gavage of 2 g glucose/kg lean body mass (25% *w/v* glucose solution). Unpaired *t*-test was used for the NC versus the HFD comparisons (**a**–**d**) and a two-way repeated measure ANOVA was used for the OGTT (**e**). Graphs indicate mean ± SD. * = $p < 0.05$. *** $p < 000.1$.

To determine the protein expression of Zip7 in NC versus HFD-fed mice, we performed western blots on proteins extracted from quadricep skeletal muscle tissue from both cohorts. We observed a significant decrease in the expression of Zip7 and Glut4 in the HFD mice groups relative to control mice (Figure 7a,b).

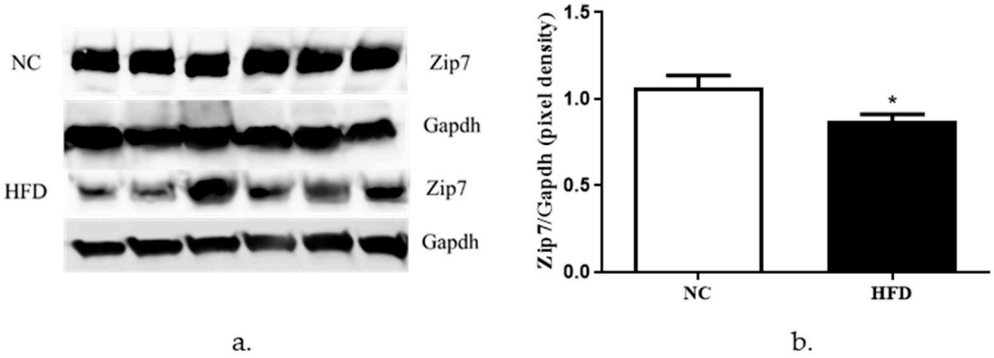

Figure 7. Analysis of Zip7 in mouse skeletal muscle tissues in NC versus HFD mice. Gapdh was used as an internal loading control and levels of Zip7 were normalized to Gapdh. (**a**) Western blot for Zip7 in NC versus HFD. (**b**) Densitometry of the western blot results. The experiments were performed on six animals per group ($n = 6$) and statistical significance was compared between the HFD to the NC mice group. * = $p < 0.05$.

We also measured the protein levels of Glut4 in the NC versus the HFD mice. We observed a significant reduction in the protein levels of Glut4 in the HFD mice when compared to the NC mice (Figure 8a,b).

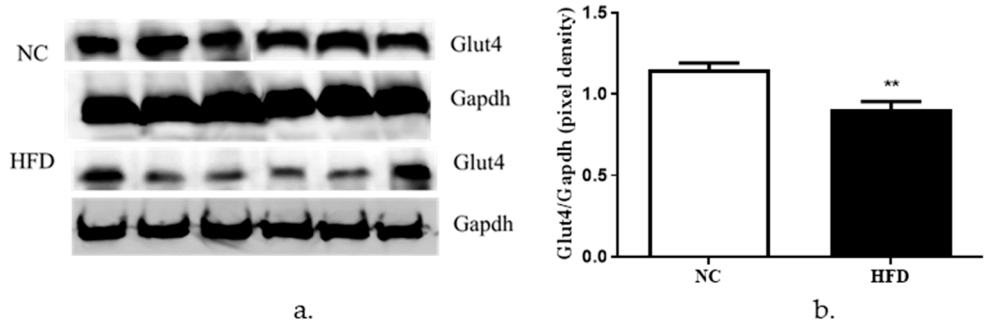

Figure 8. Analysis of Glut4 in NC versus HFD mouse skeletal muscle tissues. Gapdh was used as an internal loading control and levels of Glut4 were normalized to Gapdh. (**a**) Western blot for Glut4 in NC mice versus HFD. (**b**) Densitometry of the western blot results. The experiments were performed on six animals per group ($n = 6$) and statistical significance was compared between the HFD to the NC mice group. ** = $p < 0.01$.

4. Discussion

We have previously demonstrated the effect of zinc as an essential trace element implicated in the insulin signaling pathway [17]. However, its mechanism of action in the insulin signaling pathway and glucose uptake has not been determined. Insulin resistance, a hallmark of T2D [23], is characterized by compromised insulin-mediated activation of the PI3K/Akt pathway regulating glucose uptake via Glut4 transporters [24]. Skeletal muscle is the major site of peripheral insulin resistance of which much of the complexity of this disorder remains undefined, including the role of zinc and zinc transporter Zip7 in insulin signaling and glucose metabolism. Previous investigations showed that zinc has insulin mimetic activity leading to increased total glucose consumption [17]. Glucose transporters are important proteins that regulate glucose uptake through cellular membranes.

In this regard, Glut4 facilitates the transport of glucose in skeletal muscle [25]. Glut4 translocation to the cell membrane from specialized storage vesicles in the cytosol by insulin is a rate-limiting step of glucose disposal [26]. Accordingly, treatment of L6 rat skeletal muscle cells with zinc induced Glut4 translocation in a dose-dependent manner [26]. These studies showed that zinc exerted insulin-like effects by phosphorylation of Akt and subsequent mobilization of glut4.

Several studies have shown that disturbances in zinc transporter function and consequently zinc signaling lead to cellular and physiological disturbances. For example, zinc transporter ZnT8-deficient mice exposed to a high-fat diet develop severe insulin resistance and obesity [27]. Similarly, Zip13-deficient mice have enhanced beige adipocyte production and energy expenditure and this was concomitant with resistance to HFD-induced obesity, and improved glucose and insulin tolerance [28]. Other studies have shown that ZIP7 (and ZIP6) play an important role in regulating zinc homeostasis in MIN6 pancreatic beta cells [29]. These studies identified a compensatory increase in the expression of ZIP7 in the presence of a targeted ZIP6 knockdown. It was suggested that ZIP6 and ZIP7 transporters orchestrate cytosolic zinc flux by increasing extracellular zinc uptake or by releasing ER-stored zinc into the cytosol when required. This is supported by studies where ZIP7 knockdown in human osteosarcoma (MG-63) cells exhibited an increase in ER zinc and a decrease in cytosolic zinc [13]. Moreover, other studies have identified a dual role for ZIP7 and ZnT7 in cardiomyocytes [16]. Here it was identified that changes in the expression of ZIP7 and ZnT7 induces ER stress through the loss of ER zinc during hyperglycemic conditions. These authors also suggest that perturbations in the expression of ZIP7 and ZnT7 may lead to decompartmentalization of zinc across the ER and therefore lead to persistent ER stress.

In the context of Zip7, ablation of this transporter in mouse skeletal muscle cells resulted in reduced Glut4 protein and a reduction in insulin-stimulated glycogen synthesis [15]. To expand on this study, we examined the protein levels of both Zip7 and Glut4 in an *in vitro* insulin-resistant skeletal muscle cell model and an *in vivo* skeletal muscle of mice fed normal chow (NC) versus a high-fat diet (HFD).

First, we treated C2C12 skeletal muscle mouse cells with glucose to recapitulate hyperglycemic conditions and measured the protein levels of Zip7. We observed a significant increase in the protein levels of Zip7 in cells treated with increasing concentrations of glucose. This result is supported by studies showing high glucose induces the expression of Zip7 mRNA in mouse pancreatic islets [20] and Zip7 mRNA and protein in rat cardiomyocytes [16].

Second, we aimed to recapitulate an in vitro insulin-resistance cell culture model and investigate the protein levels of Glut4 and Zip7. C2C12 skeletal muscle cells were treated with HNMPA-(AM)3, an inhibitor of insulin receptor tyrosine kinase activity, and palmitate. HNMPA-(AM)3 abolished the expression of insulin-induced pAkt and is supported by previous studies [17,30,31]. We observed a clear reduction in Zip7 and Glut4 protein upon treatment with HNMPA-(AM)3. To our knowledge no data exists showing insulin receptor inhibition action on Zip7 expression. Similarly, palmitate-induced insulin resistance in C2C12 skeletal muscle cells was comparable with the insulin receptor inhibition study. Excessive plasma free fatty acids are associated with insulin resistance in both diabetic and non-diabetic subjects [19]. Palmitate plays a critical role in the initiation and development of insulin resistance as exposure of C2C12 cells to palmitate suppressed insulin-stimulated Akt1 phosphorylation and glucose uptake [19]. It was observed that palmitate treatment reduced insulin-induced Akt phosphorylation that is consistent with an insulin resistant state [21]. Moreover, the protein expression of Zip7 and Glut4 were reduced by palmitate and by HNMPA(AM)3. We also detected a decrease in the phospho-tyrosine expression in palmitate-treated C2C12 cells. These data suggest that Zip7 is involved in the insulin signaling pathway and glucose metabolism. While it is unclear how Zip7 contributes to the control of these pathways, there is some evidence on the potential mechanisms. For example, studies in C2C12 mouse skeletal muscle cells identified that ablation of Zip7 resulted in the modulation of key genes implicated in glucose metabolism including those of glycolysis, gluconeogenesis, the citric acid cycle, and glycogen metabolism [15]. Moreover, a reduction in the mRNA of the insulin

receptor and insulin receptor substrates 1 and 2, Glut4 protein, pAkt and insulin-induced glycogen synthesis was observed.

While these studies suggest that ZIP7 is associated with the insulin-signaling axis, how this association occurs in unclear. The role of ZIP7 in initiating zinc flux from the ER into the cytosol poses several questions, and are therefore limitations of this manuscript. For example, what are the cellular concentrations of zinc during treatment with palmitate or HNMPA-(AM)3 in an insulin-resistant state? To our knowledge, there are limited studies addressing the effect of fatty acids on cellular zinc status. One study identified the fatty acids oleate, elaidate, stearate and palmitate increased zinc concentrations in human macrophages, with elaidate having the greatest effect [32]. The authors suggest the importance of this based on zinc being a major regulator of macrophage activity. Accordingly, it will be important to determine cellular zinc status in insulin resistant skeletal muscle cells and determine whether zinc signaling processes are compromised. Moreover, the role of ZIP7 in these processes needs to be addressed. For example, if ZIP7 is reduced (or defective) in an insulin-resistant state, what effect does this have in zinc mimetic activity, and glucose metabolism?

To further investigate effects of Zip7 on glucose metabolism and insulin signaling, we established a Zip7 overexpression cell culture model. Previous studies identified that a reduction in Zip7 in skeletal muscle cells resulted in changes in several genes involved in glucose metabolism [15]. To extend on these studies, a plasmid-based system that overexpressed Zip7 was utilized to determine the ability of this transporter to regulate genes involved in the insulin signaling pathway. These studies identified significant changes in several genes including *Akt3*, *Dok2*, *Fos*, *Hras*, *Kras*, *Nos2*, *Pck2*, and *Pparg*.

It was observed that *Akt3*, *Pck2*, and *Pparg* were significantly downregulated in the Zip7-overexpressing cells. The roles of Akt1 and Akt2 are well established in metabolism, however there is less known about Akt3 [33]. It is suggested that Akt3 may play a role in defects in insulin signaling that occur in insulin resistant states [34].

There are two Pck isoenzymes, phosphoenolpyruvate carboxykinase (PEPCK-C, cytoplasmic) encoded by *Pck1* and PEPCK-M (mitochondrial), encoded by *Pck2* (mitochondrial). Although the role of *Pck1* has been extensively studied for its role in gluconeogenesis in the liver, *Pck2* remains somewhat unknown [35]. However, these data for *Pck2* are consistent with previous studies where a reduction in Zip7 mRNA led to an increase in this gene in C2C12 skeletal muscle cells [15].

Pparg (peroxisome proliferator activated receptor gamma) is a member of the nuclear hormone superfamily of transcription factors that modulates genes involved in insulin signaling and lipid metabolism [36]. While *Pparg* expression is low in skeletal muscle, C2C12 skeletal muscle cells stably overexpressing Pparg resulted in increased glucose uptake and inhibition of *Pparg* expression induced insulin resistance as determined by reduced deoxyglucose uptake [36]. In contrast, mice heterozygous for $Pparg^{+/-}$ displayed greater insulin sensitivity than the wild-type animals [37] and improved insulin resistance and obesity [38]. While these studies counter the current dogma on Pparg action in metabolism, it is not clear why *Pparg* expression is reduced in our overexpression Zip7 studies. A recent study identified Zip7 was upregulated by ethanol exposure in the livers of mice and this was concomitant with a downregulation of another *Ppar* family member, *Ppara* [39]. In C2C12 skeletal muscle cells with an ablation of *Pparg*, it was identified that myogenic differentiation was compromised [40]. These studies suggest that *Pparg* is critical for the differentiation of skeletal muscle myoblast to myotubes and perhaps Zip7 overexpression overrides the mechanism of Pparg-inhibitory effect on the myogenic phenotype. Support for a Zip7-mediated role in promoting myogenic differentiation was recently identified [41]. It was identified that zinc promotes myoblast proliferation and differentiation of mature myotubes via Zip7 activation and the modulation of the Pi3k/Akt pathway in C2C12 skeletal muscle cells.

We also observed significant upregulation of several genes in the Zip7 overexpression cell model including *Dok2*, *Fos*, *Hras*, *Kras*, and *Nos2.* Dok2 (docking protein 2), along with its closest homologue Dok1 work as adapter proteins that recruit and assemble multiple SH2-containing molecules including p120 rasGAP and Nck [42]. There is little information on the role of Dok2 in skeletal muscle or

insulin resistance, however experiments in mice lacking Dok1 or Dok2 showed enhanced expression of Erk and Akt in myeloid cells [43]. Similarly, Dok2 significantly inhibited insulin-stimulated Akt phosphorylation in COS7 cells and these authors suggested that Dok2 acts as an inhibitor of insulin action [44]. These data therefore suggest that a reduction in Dok2 would have an enhancing effect on insulin signaling.

c-Fos (FBJ osteosarcoma oncogene) is a protooncogene that dimerizes with c-Jun to form the activator protein AP1 transcription complex [45]. C-Fos is implicated in several molecular mechanisms that contribute to cellular processes including proliferation, differentiation, and apoptosis [46]. Recently, parathyroid hormone-related peptide (PTHrP) induced *c-Fos* expression was significantly reduced in Zip14-KO chondrocytes [47]. In chondrocyte differentiation, PTHrP induces phosphorylation of cAMP and the subsequent regulation of *c-Fos* via phosphorylation of the cAMP response element-binding protein (CREB) [47]. These studies suggest that Zip14 controls basal cAMP levels and its role may provide a mechanism for the zinc-mediated regulation of endocrine signaling. Whether Zip7 controls similar cAMP-mediated events (or whether Zip7 is regulated by cAMP) in skeletal muscle is yet to be determined. Zinc itself affects a wide range of second messenger and signaling molecules including Ca^{2+}/calmodulin-dependent protein kinase II [48], Erk1/2, protein tyrosine phosphatase [17] and cAMP-dependent protein kinase [49]. Future studies eliminating Zip7 in skeletal muscle for example could provide valuable insight into the specific pathways controlled by Zip7-mediated zinc flux.

We also identified an increase in the expression of *Hras* (Harvey rat sarcoma virus oncogene) and *Kras* (Kirsten rat sarcoma viral oncogene homolog) in the Zip7 overexpressing skeletal muscle cell lines. *Hras* and *Kras* are canonical *ras* genes and are critical components of cell signaling pathways that control proliferation, survival, and differentiation [50]. Ras activity induces Raf and subsequent regulation of protein kinases Mek1/2 which phosphorylate ERK1/2. Mostly, the Ras/Raf/Mek/ERK pathway is critical for cell proliferation and differentiation [51].

Finally, we assayed the expression levels of Zip7 in an *in vivo* HFD mouse model. Our results revealed a decrease in the protein expression of both Zip7 and Glut4 in the skeletal muscle of HFD-fed mice compared to the NC controls. While there are no other studies reporting this observation for reduced Zip7 in skeletal muscle of HFD-fed mice, studies on obesity-associated inflammation in mammary gland tissue found HFD mice had decreased levels of Zip7. These authors suggest that inflammation in mammary tissue compromises lactation by mediating zinc retention in the endoplasmic reticulum [52]. However, several studies have assessed zinc in the context of HFD. For example, male rats fed a HFD had reduced weight gain, abdominal fat pads, plasma insulin levels, leptin and triglycerides when supplemented with zinc [53]. Similarly, it was identified that zinc supplementation improved glucose tolerance, HOMA-β, and glucose-stimulated insulin secretion in HFD mice by enhancing pancreatic β-cell function [54]. In the context of plasma zinc levels, it has been identified in obese humans and mice that leptin levels are increased with concomitant reduced plasma zinc concentrations [55]. It was suggested by these authors that reduced zinc compromises several cell signaling pathways that regulate inflammation and leptin. This is significant given that zinc-deficiency is common in obese individuals [56].

While, the connection between Zip7 and Glut4 regulation is not known, our previous data showing a reduction in Zip7 in C2C12 skeletal muscle cells led to a significant decrease in Glut4 protein and compromised insulin-mediated glycogen synthesis [15] suggests that Zip7 might be involved in skeletal muscle glycemic control. However, the mechanisms involved in these processes requires further investigation.

The studies presented suggest that Zip7 has a role in modulating insulin-signaling molecules and is reduced in an insulin-resistant state and in mice fed a HFD. While there were several changes in genes associated with Zip7 overexpression, it is unknown how these changes contribute to insulin signaling and cell signaling processes and affect glycemic control in skeletal muscle. Further studies are required to delineate the molecular mechanisms of Zip7 action on cell signaling in both insulin-resistant and diabetic in vitro and in vivo models.

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References

1. Guariguata, L.; Whiting, D.R.; Hambleton, I.; Beagley, J.; Linnenkamp, U.; Shaw, J.E. Global estimates of diabetes prevalence for 2013 and projections for 2035. *Diabetes Res. Clin. Pract.* **2014**, *103*, 137–149. [CrossRef] [PubMed]
2. Wild, S.; Roglic, G.; Green, A.; Sicree, R.; King, H. Global Prevalence of Diabetes: Estimates for the year 2000 and projections for 2030. *Diabetes Care* **2004**, *27*, 1047–1053. [CrossRef] [PubMed]
3. Hajer, G.R.; van Haeften, T.W.; Visseren, F.L.J. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *Eur. Heart J.* **2008**, *29*, 2959–2971. [CrossRef] [PubMed]
4. Marcadenti, A.; Fuchs, S.C.; Moreira, L.B.; Wiehe, M.; Gus, M.; Fuchs, F.D. Accuracy of Anthropometric Indexes of Obesity to Predict Diabetes Mellitus Type 2 among Men and Women with Hypertension. *Am. J. Hypertens.* **2011**, *24*, 175–180. [CrossRef] [PubMed]
5. Berumen, J.; Orozco, L.; Betancourt-Cravioto, M.; Gallardo, H.; Zulueta, M.; Mendizabal, L.; Simon, L.; Benuto, R.E.; Ramírez-Campos, E.; Marin, M. Influence of obesity, parental history of diabetes, and genes in type 2 diabetes: A case-control study. *Sci. Rep.* **2019**, *9*, 2748. [CrossRef] [PubMed]
6. Janikiewicz, J.; Hanzelka, K.; Kozinski, K.; Kolczynska, K.; Dobrzyn, A. Islet β-cell failure in type 2 diabetes—Within the network of toxic lipids. *Biochem. Biophys. Res. Commun.* **2015**, *460*, 491–496. [CrossRef] [PubMed]
7. Myers, S.A.; Nield, A.; Myers, M. Zinc Transporters, Mecahnisms of Action and Therapeutic Utility: Implications for Type 2 Diabetes Mellitus. *J. Nutr. Metab.* **2012**. [CrossRef]
8. Kambe, T.; Hashimoto, A.; Fujimoto, S. Current understanding of ZIP and ZnT zinc transporters in human health and diseases. *Cell. Mol. Life Sci.* **2014**, *71*, 3281–3295. [CrossRef]
9. Kambe, T.; Tsuji, T.; Hashimoto, A.; Itsumura, N. The physiological, biochemical, and molecular roles of zinc transporters in zinc homeostasis and metabolism. *Physiol. Rev.* **2015**, *95*, 749–784. [CrossRef]
10. Cousins, R.J.; Liuzzi, J.P.; Lichten, L.A. Mammalian zinc transport, trafficking, and signals. *JBC* **2006**, *281*, 24085–24089. [CrossRef]
11. Taylor, K.M.; Hiscox, S.; Nicholson, R.I.; Hogstrand, C.; Kille, P. Protein kinase CK2 triggers cytosolic zinc signaling pathways by phosphorylation of zinc channel ZIP7. *Sci. Signal.* **2012**, *5*, ra11. [CrossRef] [PubMed]
12. Hogstrand, C.; Kille, P.; Nicholson, R.I.; Taylor, K.M. Zinc transporters and cancer: A potential role for ZIP7 as a hub for tyrosine kinase activation. *Trends Mol. Med.* **2009**, *15*, 101–111. [CrossRef] [PubMed]
13. Woodruff, G.; Bouwkamp, C.G.; de Vrij, F.M.; Lovenberg, T.; Bonaventure, P.; Kushner, S.A.; Harrington, A.W. The Zinc Transporter SLC39A7 (ZIP7) Is Essential for Regulation of Cytosolic Zinc Levels. *Mol. Pharm.* **2018**, *94*, 1092–1100. [CrossRef] [PubMed]
14. Taylor, K.M.; Vichova, P.; Jordan, N.; Hiscox, S.; Hendley, R.; Nicholson, R.I. ZIP7-Mediated Intracellular Zinc Transport Contributes to Aberrant Growth Factor Signaling in Antihormone-Resistant Breast Cancer Cells. *Endocrinology* **2008**, *149*, 4912–4920. [CrossRef] [PubMed]
15. Myers, S.A.; Nield, A.; Chew, G.S.; Myers, M.A. The zinc transporter, Slc39a7 (Zip7) is implicated in glycaemic control in skeletal muscle cells. *PLoS ONE* **2013**, *8*, e79316. [CrossRef]
16. Tuncay, E.; Bitirim, V.C.; Durak, A.; Carrat, G.R.J.; Taylor, K.M.; Rutter, G.A.; Turan, B. Hyperglycemia-Induced Changes in ZIP7 and ZnT7 Expression Cause Zn(2+) Release From the Sarco(endo)plasmic Reticulum and Mediate ER Stress in the Heart. *Diabetes* **2017**, *66*, 1346–1358. [CrossRef]

17. Norouzi, S.; Adulcikas, J.; Sohal, S.S.; Myers, S. Zinc stimulates glucose oxidation and glycemic control by modulating the insulin signaling pathway in human and mouse skeletal muscle cell lines. *PLoS ONE* **2018**, *13*, e0191727. [CrossRef]
18. Marshall, J.P.S.; Estevez, E.; Kammoun, H.L.; King, E.J.; Bruce, C.R.; Drew, B.G.; Qian, H.; Iliades, P.; Gregorevic, P.; Febbraio, M.A.; et al. Skeletal muscle-specific overexpression of heat shock protein 72 improves skeletal muscle insulin-stimulated glucose uptake but does not alter whole body metabolism. *Diabetes Obes. Metab.* **2018**, *20*, 1928–1936. [CrossRef]
19. Yang, M.; Wei, D.; Mo, C.; Zhang, J.; Wang, X.; Han, X.; Wang, Z.; Xiao, H. Saturated fatty acid palmitate-induced insulin resistance is accompanied with myotube loss and the impaired expression of health benefit myokine genes in C2C12 myotubes. *Lipids Health Dis.* **2013**, *12*, 104. [CrossRef]
20. Bellomo, E.A.; Meur, G.; Rutter, G.A. Glucose Regulates Free Cytosolic Zn^{2+} Concentration, Slc39 (ZiP), and Metallothionein Gene Expression in Primary Pancreatic Islet β-Cells. *J. Biol. Chem.* **2011**, *286*, 25778–25789. [CrossRef]
21. Feng, X.T.; Wang, T.Z.; Leng, J.; Chen, Y.; Liu, J.B.; Liu, Y.; Wang, W.J. Palmitate contributes to insulin resistance through downregulation of the Src-mediated phosphorylation of Akt in C2C12 myotubes. *Biosci. Biotechnol. Biochem.* **2012**, *76*, 1356–1361. [CrossRef] [PubMed]
22. Nakamura, S.; Takamura, T.; Matsuzawa-Nagata, N.; Takayama, H.; Misu, H.; Noda, H.; Nabemoto, S.; Kurita, S.; Ota, T.; Ando, H.; et al. Palmitate induces insulin resistance in H4IIEC3 hepatocytes through reactive oxygen species produced by mitochondria. *J. Biol. Chem.* **2009**, *284*, 14809–14818. [CrossRef] [PubMed]
23. Taylor, R. Insulin Resistance and Type 2 Diabetes. *Diabetes* **2012**, *61*, 778–779. [CrossRef] [PubMed]
24. Bevan, P. Insulin signalling. *J. Cell Sci.* **2001**, *114*, 1429–1430. [PubMed]
25. Tokarz, V.L.; MacDonald, P.E.; Klip, A. The cell biology of systemic insulin function. *J. Cell Biol.* **2018**. [CrossRef] [PubMed]
26. Wu, Y.; Lu, H.; Yang, H.; Li, C.; Sang, Q.; Liu, X.; Liu, Y.; Wang, Y.; Sun, Z. Zinc stimulates glucose consumption by modulating the insulin signaling pathway in L6 myotubes: Essential roles of Akt–GLUT4, GSK3β and mTOR–S6K1. *J. Nutr. Biochem.* **2016**, *34*, 126–135. [CrossRef]
27. Hardy, A.B.; Wijesekara, N.; Genkin, I.; Prentice, K.J.; Bhattacharjee, A.; Kong, D.; Chimienti, F.; Wheeler, M.B. Effects of high-fat diet feeding on Znt8-null mice: Differences between beta-cell and global knockout of Znt8. *Am. J. Physiol. Endocrinol. Metab.* **2012**, *302*, E1084–E1096. [CrossRef]
28. Fukunaka, A.; Fukada, T.; Bhin, J.; Suzuki, L.; Tsuzuki, T.; Takamine, Y.; Bin, B.-H.; Yoshihara, T.; Ichinoseki-Sekine, N.; Naito, H.; et al. Zinc transporter ZIP13 suppresses beige adipocyte biogenesis and energy expenditure by regulating C/EBP-β expression. *PLoS Genet.* **2017**, *13*, e1006950. [CrossRef]
29. Liu, Y.; Batchuluun, B.; Ho, L.; Zhu, D.; Prentice, K.J.; Bhattacharjee, A.; Zhang, M.; Pourasgari, F.; Hardy, A.B.; Taylor, K.M. Characterization of Zinc Influx Transporters (ZIPs) in Pancreatic β Cells Roles in Regulating Cytosolic Zinc Homeostasis and Insulin Secretion. *J. Biol. Chem.* **2015**, *290*, 18757–18769. [CrossRef]
30. Jewell, J.L.; Oh, E.; Ramalingam, L.; Kalwat, M.A.; Tagliabracci, V.S.; Tackett, L.; Elmendorf, J.S.; Thurmond, D.C. Munc18c phosphorylation by the insulin receptor links cell signaling directly to SNARE exocytosis. *J. Cell Biol.* **2011**, *193*, 185–199. [CrossRef]
31. Song, E.K.; Lee, Y.R.; Kim, Y.R.; Yeom, J.H.; Yoo, C.H.; Kim, H.K.; Park, H.M.; Kang, H.S.; Kim, J.S.; Kim, U.H.; et al. NAADP mediates insulin-stimulated glucose uptake and insulin sensitization by PPARgamma in adipocytes. *Cell Rep.* **2012**, *2*, 1607–1619. [CrossRef] [PubMed]
32. Zacherl, J.R.; Tourkova, I.; St Croix, C.M.; Robinson, L.J.; Peck Palmer, O.M.; Mihalik, S.J.; Blair, H.C. Elaidate, an 18-carbon trans-monoenoic fatty acid, but not physiological fatty acids increases intracellular Zn(2+) in human macrophages. *J. Cell. Biochem.* **2015**, *116*, 524–532. [CrossRef] [PubMed]
33. Ding, L.; Zhang, L.; Biswas, S.; Schugar, R.C.; Brown, J.M.; Byzova, T.; Podrez, E. Akt3 inhibits adipogenesis and protects from diet-induced obesity via WNK1/SGK1 signaling. *JCI Insight* **2017**, *2*, e95687. [CrossRef] [PubMed]

34. Brozinick, J.T.; Roberts, B.R.; Dohm, G.L. Defective Signaling Through Akt-2 and -3 but Not Akt-1 in Insulin-Resistant Human Skeletal Muscle. *Potential Role Insul. Resist.* **2003**, *52*, 935–941.
35. Brown, D.M.; Williams, H.; Ryan, K.J.; Wilson, T.L.; Daniel, Z.C.; Mareko, M.H.; Emes, R.D.; Harris, D.W.; Jones, S.; Wattis, J.A.; et al. Mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M) and serine biosynthetic pathway genes are co-ordinately increased during anabolic agent-induced skeletal muscle growth. *Sci. Rep.* **2016**, *6*, 28693. [CrossRef] [PubMed]
36. Verma, N.K.; Singh, J.; Dey, C.S. PPAR-gamma expression modulates insulin sensitivity in C2C12 skeletal muscle cells. *Br. J. Pharmacol.* **2004**, *143*, 1006–1013. [CrossRef] [PubMed]
37. Miles, P.D.; Barak, Y.; He, W.; Evans, R.M.; Olefsky, J.M. Improved insulin-sensitivity in mice heterozygous for PPAR-gamma deficiency. *J. Clin. Investig.* **2000**, *105*, 287–292. [CrossRef] [PubMed]
38. Yamauchi, T.; Kamon, J.; Waki, H.; Murakami, K.; Motojima, K.; Komeda, K.; Ide, T.; Kubota, N.; Terauchi, Y.; Tobe, K.; et al. The mechanisms by which both heterozygous peroxisome proliferator-activated receptor gamma (PPARgamma) deficiency and PPARgamma agonist improve insulin resistance. *J. Biol. Chem.* **2001**, *276*, 41245–41254. [CrossRef]
39. Sun, Q.; Li, Q.; Zhong, W.; Zhang, J.; Sun, X.; Tan, X.; Yin, X.; Sun, X.; Zhang, X.; Zhou, Z. Dysregulation of hepatic zinc transporters in a mouse model of alcoholic liver disease. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2014**, *307*, G313–G322. [CrossRef]
40. Singh, J.; Verma, N.K.; Kansagra, S.M.; Kate, B.N.; Dey, C.S. Altered PPARgamma expression inhibits myogenic differentiation in C2C12 skeletal muscle cells. *Mol. Cell. Biochem.* **2007**, *294*, 163–171. [CrossRef]
41. Mnatsakanyan, H.; i Serra, R.S.; Rico, P.; Salmerón-Sánchez, M. Zinc uptake promotes myoblast differentiation via Zip7 transporter and activation of Akt signalling transduction pathway. *Sci. Rep.* **2018**, *8*, 13642. [CrossRef] [PubMed]
42. Shinohara, H.; Inoue, A.; Toyama-Sorimachi, N.; Nagai, Y.; Yasuda, T.; Suzuki, H.; Horai, R.; Iwakura, Y.; Yamamoto, T.; Karasuyama, H.; et al. Dok-1 and Dok-2 are negative regulators of lipopolysaccharide-induced signaling. *J. Exp. Med.* **2005**, *201*, 333–339. [CrossRef] [PubMed]
43. Yasuda, T.; Shirakata, M.; Iwama, A.; Ishii, A.; Ebihara, Y.; Osawa, M.; Honda, K.; Shinohara, H.; Sudo, K.; Tsuji, K.; et al. Role of Dok-1 and Dok-2 in myeloid homeostasis and suppression of leukemia. *J. Exp. Med.* **2004**, *200*, 1681–1687. [CrossRef] [PubMed]
44. Huang, S.M.; Hancock, M.K.; Pitman, J.L.; Orth, A.P.; Gekakis, N. Negative regulators of insulin signaling revealed in a genome-wide functional screen. *PLoS ONE* **2009**, *4*, e6871. [CrossRef] [PubMed]
45. Tu, Y.C.; Huang, D.Y.; Shiah, S.G.; Wang, J.S.; Lin, W.W. Regulation of c-Fos gene expression by NF-kappaB: A p65 homodimer binding site in mouse embryonic fibroblasts but not human HEK293 cells. *PLoS ONE* **2013**, *8*, e84062. [CrossRef]
46. Delghandi, M.P.; Johannessen, M.; Moens, U. The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. *Cell Signal.* **2005**, *17*, 1343–1351. [CrossRef] [PubMed]
47. Hojyo, S.; Fukada, T.; Shimoda, S.; Ohashi, W.; Bin, B.H.; Koseki, H.; Hirano, T. The zinc transporter SLC39A14/ZIP14 controls G-protein coupled receptor-mediated signaling required for systemic growth. *PLoS ONE* **2011**, *6*, e18059. [CrossRef]
48. Yan, G.; Zhang, Y.; Yu, J.; Yu, Y.; Zhang, F.; Zhang, Z.; Wu, A.; Yan, X.; Zhou, Y.; Wang, F. Slc39a7/zip7 plays a critical role in development and zinc homeostasis in zebrafish. *PLoS ONE* **2012**, *7*, e42939. [CrossRef]
49. Fukada, T.; Yamasaki, S.; Nishida, K.; Murakami, M.; Hirano, T. Zinc homeostasis and signaling in health and diseases. *J. Biol. Inorg. Chem.* **2011**, *16*, 1123–1134. [CrossRef]
50. Castellano, E.; Santos, E. Functional specificity of ras isoforms: So similar but so different. *Genes Cancer* **2011**, *2*, 216–231. [CrossRef]
51. Klein, C.; Creach, K.; Irintcheva, V.; Hughes, K.J.; Blackwell, P.L.; Corbett, J.A.; Baldassare, J.J. Zinc induces ERK-dependent cell death through a specific Ras isoform. *Apoptosis Int. J. Program. Cell Death* **2006**, *11*, 1933–1944. [CrossRef] [PubMed]
52. Hennigar, S.R.; Velasquez, V.; Kelleher, S.L. Obesity-Induced Inflammation Is Associated with Alterations in Subcellular Zinc Pools and Premature Mammary Gland Involution in Lactating Mice. *J. Nutr.* **2015**, *145*, 1999–2005. [CrossRef] [PubMed]
53. Tinkov, A.A.; Popova, E.V.; Gatiatulina, E.R.; Skalnaya, A.A.; Yakovenko, E.N.; Alchinova, I.B.; Karganov, M.Y.; Skalny, A.V.; Nikonorov, A.A. Decreased adipose tissue zinc content is associated with metabolic parameters in high fat fed Wistar rats. *Acta Sci. Pol. Technol. Aliment.* **2016**, *15*, 99–105. [CrossRef] [PubMed]

54. Cooper-Capetini, V.; de Vasconcelos, D.A.A.; Martins, A.R.; Hirabara, S.M.; Donato, J., Jr.; Carpinelli, A.R.; Abdulkader, F. Zinc Supplementation Improves Glucose Homeostasis in High Fat-Fed Mice by Enhancing Pancreatic beta-Cell Function. *Nutrients* **2017**, *9*, 1150. [CrossRef] [PubMed]
55. Liu, M.J.; Bao, S.; Bolin, E.R.; Burris, D.L.; Xu, X.; Sun, Q.; Killilea, D.W.; Shen, Q.; Ziouzenkova, O.; Belury, M.A.; et al. Zinc deficiency augments leptin production and exacerbates macrophage infiltration into adipose tissue in mice fed a high-fat diet. *J. Nutr.* **2013**, *143*, 1036–1045. [CrossRef] [PubMed]
56. Di Martino, G.; Matera, M.G.; De Martino, B.; Vacca, C.; Di Martino, S.; Rossi, F. Relationship between zinc and obesity. *J. Med.* **1993**, *24*, 177–183. [PubMed]

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CHAPTER 6

GENERAL DISCUSSION

6.1. Final Discussion

The overall aim of this thesis was to identify the role of zinc and the zinc transporter Zip7 in modulating the insulin signalling pathway and glucose metabolism in skeletal muscle cells. Zinc is an essential metal ion implicated in several biological processes and dysfunctional zinc signalling is associated with various disease states (1). The results from these studies suggest that zinc induces cell signalling molecules associated with glucose homeostasis in skeletal muscle. The subsequent zinc-mediated phosphorylation of cell signalling molecules associated with the insulin-signalling axis and increased glucose oxidation suggests that both insulin and zinc are critically important in maintaining glucose homeostasis in skeletal muscle (1).

The insulin-like activity of zinc in processes involved in cellular function and homeostasis suggests that aberrant levels of zinc, and decompartmentalisation of zinc will have biological effects that could be amendable to clinical intervention. Zinc transporter proteins are implicated in several cell processes that facilitate insulin signalling and glucose control and therefore could offer exciting new molecular targets that have utility for clinical intervention in the treatment of IR and T2DM and other diseases associated with compromised metabolism. According to several studies on Zip7 as the "gate-keeper" of zinc release from subcellular organelles and subsequent Zip7-mediated cell signalling events in skeletal muscle, no doubt place this transporter in an important position for further studies (2).

In this thesis it was identified that zinc has insulin-mimetic-like activity in skeletal muscle through the activation of several cell signalling molecules and the concomitant mobilisation of glucose uptake. Moreover, it was observed that the Zip7 protein is diminished in pharmacologically and fatty acid-induced insulin-resistant mouse skeletal muscle cells and in mice fed a HFD. Moreover, the overexpression of Zip7 protein in mouse skeletal muscle cells regulated the modulation of several key insulin signalling molecules. Therefore, these studies now provide an important process to further delineate the molecular mechanisms of zinc and Zip7 in the context of IR and T2DM. These results also provide a significant advancement in knowledge contributing to the emerging concept and paradigm that zinc transporters are important factors involved in maintaining physiological zinc processes and cellular homeostasis and that dysfunctional zinc signalling contributes to insulin resistance and T2DM progression. These key findings are novel and will add to the current scientific knowledge of

zinc and the zinc transporter Zip7 in the context of mediating cell signalling activity in skeletal muscle and therefore may contribute to the discovery of novel treatments for IR and T2DM.

6.2. Future research

The broad nature of insulin resistance in skeletal muscle suggests a defect in the proximal component of the insulin signalling network. Given the unique role of zinc in cellular signalling and the activity of ZIP7 as the 'gatekeeper' of zinc release, it will be important to further characterise their mechanisms of action in insulin-sensitive tissues. Some examples of future research include:

6.2.1. Inhibition studies to determine how zinc targets cell signalling molecules

In this thesis it was shown that the insulin receptor tyrosine kinase inhibitor HNMPA-(AM)3 inhibited zinc-induced Akt activation suggesting that zinc acts through the insulin signalling pathway. To extend this study, several inhibition studies can be performed to block various cell signalling molecules "upstream" of Akt, and "downstream" of the insulin receptor to determine how zinc is acting on the phosphorylation of Akt and thus glucose homeostasis. Several cell molecule inhibitors can be used in various combinations. These include LY294002 (a reversible inhibitor of PI3K) (3); Wortmannin (an irreversible inhibitor of PI3K) (3); UO126 (a selective inhibitor of MAP kinase kinases, MEK1 and MEK2) (4) and OSU-03012 (a PDK1 inhibitor) (5). These inhibitors will help "dissect" out the pathways of zinc-mediated cell signalling events. For example, inhibition of upstream molecular targets of Akt and treatment of skeletal muscle cells with zinc will elucidate whether zinc can independently activate Akt in the presence of a non-functional insulin signalling axis.

Similarly, treatment of skeletal muscle cells with a zinc chelator (e.g. TPEN) will provide an insight into whether zinc treatment can independently activate cell signalling molecules and glucose uptake over that of insulin treatment. For example, skeletal muscle cells could be treated with insulin to activate pAkt in the presence of TPEN. If pAkt is not activated by insulin in this situation, then this will suggest that zinc is required and the insulin signalling axis depends on zinc. Moreover, utilising siRNA technology to reduce the levels of Zip7 in skeletal

muscle cells and subsequently treating cells with insulin to activate the insulin signalling axis will determine if Zip7 is required for this axis.

6.2.2. Assess the 'gated' release of zinc from intracellular stores by Zip7 and subsequent activation of the insulin signalling pathway in non-insulin resistant versus insulin-resistant skeletal muscle cells.

The idea that Zip7 controls zinc flux from subcellular organelles and consequently zinc-mediated activation of cell signalling molecules presents an opportunity to further delineate the mechanisms of action of this transporter. To understand these mechanisms in more detail, insulin-resistant skeletal muscle cells can be created by chronically treating skeletal muscle cells in the presence of palmitate or pharmacologically with an insulin receptor inhibitor. Initially, non-insulin resistant control and insulin resistant skeletal muscle cells could be monitored by live cell imaging confocal microscopy for changes in cytosolic zinc concentrations by application of exogenous zinc treatment and detection with FluorZin-3 (a high affinity zinc fluorimetric probe that binds zinc). Then, changes in zinc flux could be monitored in the presence of a Zip7 overexpression or Zip7 knockdown. Following the changes in zinc flux in the presence of an overexpressing Zip7 or knock-down Zip7, changes in insulin signalling molecules could be monitored by investigating downstream targets of insulin signalling utilising antibody protein arrays and changes in glucose oxidation in both the insulin-resistant and non-insulin resistant skeletal muscle cells.

6.2.3. Assess the Zip7-mediated zinc mobilisation of Glut4 protein and subsequent glucose oxidation in non-insulin resistant versus insulin resistant skeletal muscle.

Building on from the above studies it will be important to identify the link between Zip7 and glucose oxidation through the mobilisation of Glut4 in insulin-resistant skeletal muscle. Accordingly, Glut4 mobilisation could be monitored by the construction of skeletal muscle cells stably expressing GFP epitope-tagged Glut4. Given the experimental problems of determining insulin and zinc-mediated mobilisation and translocation of Glut4 in Chapter 4, some redesign of the methods is required. Thus, the following experiments could be performed. 1). Utilisation of different cell lines that are more insulin-sensitive, such as other skeletal muscle cells and adipocytes that express Glut4. For example, 3T3-L1 fibroblasts can be effectively differentiated into adipocyte cells which expresses Glut4 and responds well to insulin (6). In cultured muscle cells, it is suggested that the acquisition of the myoblast to the

mature myotubular phenotype is not an efficient process and therefore muscle cells lack important phenotypic characteristics including lateralised nuclei, a developed tubule network and insulin sensitivity (7). Therefore, another strategy would be to obtain muscle biopsies from non-diabetic and type 2 diabetic patients and use these to culture primary skeletal muscle cell lines that would have the developed phenotypic characteristics.

Another strategy would be to use a Glut4 redistribution assay designed and manufactured by Thermo Fisher. The redistribution technology monitors the cellular translocation of GFP-tagged Glut4 in response to compounds (e.g. insulin or zinc) or other stimuli. The assay is based on receiving cell lines that already stably express the human Glut4 fused to the N-terminus of GFP. Insulin is used in this assay as a reference agonist. The assay can be used to screen for compounds that enhance the translocation of Glut4 in response to sensitisers (e.g. zinc).

The above studies could be combined with a Zip7 overexpression system or a Zip7 knock down to assess the role of this transporter in regulating, not only zinc flux, but Glut4 mobilisation and translocation to the outer plasma membrane.

6.2.4. Determine the consequence of overexpression of Zip7 or Zip7 knock down in mouse skeletal muscle tissue on modulating pathways implicated in glycaemic control and homeostasis.

While studies in cell lines *in vitro* are essential for understanding mechanisms related to zinc-mediated cell signalling processes and subsequent glucose metabolism, it will be essential to create an *in vivo* model to better understand these mechanisms in a biological and physiological context. Several experiments could be performed including *in vivo* electroporation (IVE) of Zip7 overexpression plasmid or knock-down siRNA plasmid in mouse skeletal muscle (tibialis anterior, TA). IVE is a powerful experimental approach that allows manipulation of the gene of interest in the test leg and comparison with the control leg in the same animal. Initially we could use wild-type C57Bl/6 mice for IVE of Zip7 into TA and the subsequent measurement of insulin signalling pathway proteins. Serum levels of glucose and insulin could also be measured to give an indication of whether there are any changes in response to these molecules in skeletal muscle.

6.2.5. Animal knock out studies

It will be also essential to test the Zip7 function in animal knockout and/or overexpression models. Currently the majority of knockout and overexpression studies for Zip7 have been performed in cells and there is no Zip7 knockout animal model system. Gene knockout and overexpression models represent powerful tools for studying possible functions and targets of regulatory factors involved in physiological processes (8). Given that zinc is essential for cell survival, a global knockout of Zip7 would probably have deleterious effects on the animal. Therefore, skeletal muscle-specific knockdown and or overexpression of Zip7 could be created using an adeno-associated viral vector (AVV). For example, the Zip7 recombinant AAV vector could contain a skeletal-muscle-specific creatine kinase promoter to drive the expression of Zip7 in this tissue (9). Several studies could then be performed to determine the effect of Zip7 changes on several parameters including: insulin and glucose tolerance tests, HFD induced weight gain or adiposity, metabolic parameters such as energy expenditure and physical activity. Animal models could include the db/db mice which have leptin deficiency and is currently the mostly widely used animal model of T2DM, or wild type mice fed HFD to induce adiposity and T2DM.

6.2.6. Human insulin resistance and type 2 diabetes tissue

While the above future studies to elucidate the molecular mechanisms of zinc- and Zip7-mediated signalling will be highly important, it will be essential to perform these studies in insulin resistant and type 2 diabetic human skeletal muscle samples. Skeletal muscle tissue biopsies could be collected from both prediabetic and type 2 diabetic patients. Two strategies could be used to determine the clinical relevance of zinc and Zip7 in these disorders.

1). Primary cell lines could be generated from prediabetic (insulin resistant) and type 2 diabetic skeletal muscle tissue. These cell lines could then undergo similar experiments as outlined in Chapters 3,4, and 5. Moreover, current drug therapies (e.g. metformin) could be tested in these cells to determine if zinc and/or Zip7 is required for their efficacy.

2). Skeletal muscle tissue from prediabetic and type 2 diabetic patients could be screened for the changes in the expression of the family of zinc transporters. Thus far, this has not been done. This might provide insight into other potential zinc transporters that could perhaps be useful in further studies. For example, ZnT8, the key zinc transporter expressed in the pancreas and involved in importing zinc into the beta cells where it is involved in insulin crystallisation,

has several key SNPs that compromise its function (10). Perhaps SNPs could be screened for the zinc transporters in prediabetic and type 2 diabetic skeletal muscle. While this would require large cohorts of participants, it is certainly feasible.

6.3. Limitations

In terms of limitations, the work completed in this thesis would benefit from the inclusion of other cell models. Although skeletal muscle is responsible for 70% of glucose disposal (11), it would be of value to repeat these experiments in other insulin sensitive tissue cell lines such as liver and adipose cells/tissue (12, 13) to determine if these tissues can respond to the zinc. It will be important to address this as there is considerable "cross-talk" across these tissues that work together to improve overall metabolic health (14). Thus, it is a limitation in this thesis that only skeletal muscle was used as this does not inform the greater physiological challenges associated with a whole animal model. However, reducing cellular components into single cell types (such as skeletal muscle for example) is very useful to establish molecular mechanisms that are otherwise difficult to test in whole animal studies.

Another limitation of these studies was that Zip7-mediated zinc flux was not assessed. Without these studies it is difficult to determine whether the effect observed from zinc-mediated cell signalling events was from the release of zinc from the endoplasmic reticulum by Zip7. While this is a limitation, it was not a major oversight as the thesis focus was on the molecular mechanisms of zinc-mediated activation of cell signalling molecules. However, future studies should most definitely address this.

The work in this thesis was predominately in mouse cell lines. Accordingly, it will be important to consider a human cell model of diabetes for future work. Human diabetic cells can be purchased commercially and could be used to provide a more robust, physiological model to understand molecular mechanisms of zinc and Zip7 function. Similarly, human skeletal muscle tissue and serum could be collected from type 2 diabetic patients and analysed for Zip7 (and other zinc transporters) and zinc status.

To move toward a more physiologically relevant model, it will be important to repeat these studies in animal models where many factors interact to control physiological mechanisms. In the whole animal there are many stimulatory or inhibitory pathways that act to regulate each physiological mechanism. In cell culture it is possible to study one of these at a time, but it is difficult to study all the possible interactions that can occur in physiological circumstances. The ultimate information from these studies is not the effect on isolated cells, but how the mechanism is regulated under the myriad of interactions that occur in the whole living animal (15).

6.4. Future challenges and opportunities for zinc biology and type 2 diabetes

Studies on the role of zinc and its transporters in skeletal muscle of insulin resistant and type 2 diabetics are likely to manifest novel zinc functions, as well as promising approaches for the treatment of these diseases. This thesis has discussed several critical roles of zinc and the zinc transporter Zip7 in normal and insulin resistant/type 2 diabetic skeletal muscle. Although the role of zinc transporters in IR and T2DM is not fully understood, it is clear from studies on zinc transporters that they have utility for the development of novel diabetic therapies. The results from studies on skeletal muscle are beginning to reveal that zinc and Zip7 play an important role in the processes that facilitate insulin signalling and glycaemic control and therefore, could offer exciting new targets that are amendable to therapeutic intervention in the treatment of diseases associated with IR and T2DM. Some questions are not yet solved regarding Zip7 and the target molecules of zinc signalling. These include:

- What are the mechanisms underlying the expression of Zip7? It is not clear how Zip7 is regulated. For example, how is the Zip7-mediated gated release of zinc from the endoplasmic reticulum induced?
- Are the mechanisms of Zip7-mediated zinc release dysfunctional in insulin resistance and type 2 diabetes?
- Are there changes in the expression levels of Zip7 or changes in the concentration of zinc released in these models of insulin resistance and type 2 diabetes?
- Is zinc decompartmentalised? That is, is zinc concentrations found in intracellular organelles that are not within a normal range?

- Are other zinc transporters implicated in zinc-mediated cell signalling events and glucose control in skeletal muscle (and other tissues)?
- What are the molecular mechanisms of zinc-mediated activation of cell signalling molecules involved in glucose uptake?

Elucidating novel biochemical functions of zinc and Zip7 within the cellular network of skeletal muscle will lead to improvements in the understanding of IR and potentially facilitate the development of diagnostic and effective therapeutic strategies. It should be highlighted that while current therapies to treat IR and T2DM are efficacious, some individuals do not respond to these treatments and there is associated side-effects. Thus, novel treatments targeting zinc transport in combination with current drug treatments could augment a better glycaemic response in patients. These studies could also open opportunities for the design of novel analogous of zinc that could be used as an adjunct therapy with existing drugs or used alone to improve glycaemic control in IR and T2DM.

References

1. Norouzi S, Adulcikas J, Sohal SS, Myers S. Zinc stimulates glucose oxidation and glycemic control by modulating the insulin signaling pathway in human and mouse skeletal muscle cell lines. PLoS One. 2018;13(1):e0191727.
2. Norouzi S, Adulcikas J, Sohal SS, Myers S. Zinc transporters and insulin resistance: therapeutic implications for type 2 diabetes and metabolic disease. Journal of biomedical science. 2017;24(1):87.
3. McNamara CR, Degterev A. Small-molecule inhibitors of the PI3K signaling network. Future medicinal chemistry. 2011;3(5):549-65.
4. Newton R, Cambridge L, Hart LA, Stevens DA, Lindsay MA, Barnes PJ. The MAP kinase inhibitors, PD098059, UO126 and SB203580, inhibit IL-1β-dependent PGE2 release via mechanistically distinct processes. British journal of pharmacology. 2000;130(6):1353-61.
5. Lee TX, Packer MD, Huang J, Akhmametyeva EM, Kulp SK, Chen C-S, et al. Growth inhibitory and anti-tumour activities of OSU-03012, a novel PDK-1 inhibitor, on vestibular schwannoma and malignant schwannoma cells. European journal of cancer. 2009;45(9):1709-20.
6. Govers R. Molecular mechanisms of GLUT4 regulation in adipocytes. Diabetes & metabolism. 2014;40(6):400-10.
7. Lauritzen HP. In vivo imaging of GLUT4 translocation. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme. 2009;34(3):420-3.
8. Bilezikian JP, Raisz LG, Martin TJ. Principles of bone biology: Academic press; 2008.
9. Marshall JPS, Estevez E, Kammoun HL, King EJ, Bruce CR, Drew BG, et al. Skeletal muscle-specific overexpression of heat shock protein 72 improves skeletal muscle insulin-stimulated glucose uptake but does not alter whole body metabolism. Diabetes, obesity & metabolism. 2018;20(8):1928-36.
10. Rutter GA, Chimienti F. SLC30A8 mutations in type 2 diabetes. Diabetologia. 2015;58(1):31-6.
11. DeFronzo RA, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes care. 2009;32(suppl 2):S157-S63.
12. Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. Nature. 2001;414(6865):799.
13. Ali O. Genetics of type 2 diabetes. World journal of diabetes. 2013;4(4):114.
14. Stanford KI, Goodyear LJ. Muscle-Adipose Tissue Cross Talk. Cold Spring Harbor perspectives in medicine. 2018;8(8).
15. Murphy HC. The use of whole animals versus isolated organs or cell culture in research. 1991.

APPENDICES

Appendix-1: Copies of conferences posters

UNIVERSITY of TASMANIA

Zinc is critical for the activation of cell signalling molecules in skeletal muscle: implications for insulin resistance and type 2 diabetes

Shaghayegh Norouzi and Stephen Myers
School of Health Sciences, University of Tasmania, Launceston, Australia

INTRODUCTION

Insulin resistance (IR) is a major risk factor for the development of chronic disease including type 2 diabetes mellitus (T2D) [1]. Skeletal muscle is the major site of peripheral insulin resistance and glucose intolerance [2] and is the major storage tissue for zinc ion [3]. Zinc plays an important role in glucose homeostasis through the regulation of various cell signaling pathways and evidence suggests that it acts as an insulin-mimetic in the control of cellular metabolism [4]. Zinc has emerged as potential therapeutic targets for disease states associated with dysfunctional metabolism [5]. For example, essential dietary zinc plays a major role in metabolic homeostasis in peripheral tissues that respond to insulin [6]. While zinc is an important component of maintaining cellular homeostasis; it will be important to further define its mechanisms of zinc action in regulating insulin sensitivity in peripheral tissue such as skeletal muscle.

METHODS

skeletal muscle (C2C12) cells were cultured and treated with 10 nM insulin and/or 20 µM zinc over 0-60 minutes and assayed for the phosphorylation of AKT (protein kinase B) by western blotting. The activation of Extra cellular signal-regulated kinases (ERK1/2), Proline-rich AKT substrate of 40 kDa (PRAS40) and Glycogen synthesis kinase 3 beta (GSK-3β) also detected using intracellular signalling protein array kit.

RESULTS

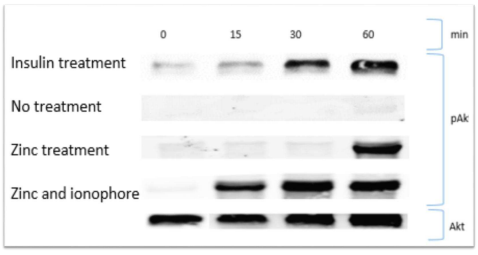

Figure 1. Western blot analysis of pAkt expressions in C2C12 cells treated with 10 nM insulin, nothing, 10 µM zinc, 10 µm zinc and ionophore, respectively.

RESULTS

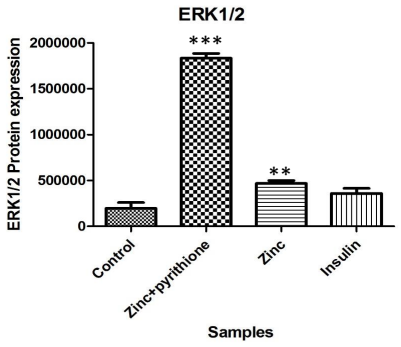

Figure 2. The results of 3 independent experiments are presented as mean ± standard error. $^{**}P < 0.01$ and $^{***}P < 0.001$ considered significant compared with Control.

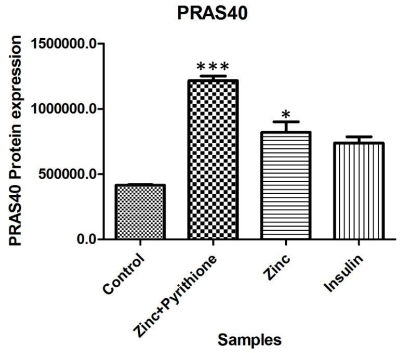

Figure 3. The results of 3 independent experiments are presented as mean ± standard error. $^{*}P < 0.05$ and $^{***}P < 0.001$ considered significant compared with Control.

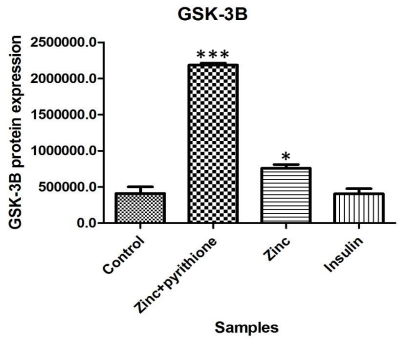

Figure 4. The results of 3 independent experiments are presented as mean ± standard error. $^{*}P < 0.05$ and $^{***}P < 0.001$ considered significant compared with Control.

DISCUSSION

We found that zinc could activate pAKT in a time-dependent manner which is consistent with the literature. Akt is an important molecule involved in activating downstream pathways of glucose mobilisation and uptake [7]. We identified that zinc also activates ERK1/2, PRAS40 and GSK-3β which they are important in cell proliferation and survival [8], insulin-stimulated mTORC [9], and glycogen synthesis [10], respectively. So, zinc activates important molecules implicated in glucose metabolism in skeletal muscle.

This study may result in fundamental outcomes that will provide a platform to help understand other organ systems and disease states. For example, many approved or being studied drugs that target zinc signalling might prove useful for the treatment of T2D or other diseases.

REFERENCES

1. Højlund, K., *Metabolism and insulin signaling in common metabolic disorders and inherited insulin resistance.* Danish medical journal, 2014. **61**(7): p. B4890-B4890.

2. Joint, F. and W.H. Organization, *Vitamin and mineral requirements in human nutrition.* 2005.

3. Lee, S.R., et al., *The critical roles of zinc: beyond impact on myocardial signaling.* The Korean Journal of Physiology & Pharmacology, 2015. **19**(5): p. 389-399.

4. Fukada, T., et al., *Zinc homeostasis and signaling in health and diseases.* JBIC Journal of Biological Inorganic Chemistry, 2011. **16**(7): p. 1123-1134.

5. Cruz, K.J.C., A.R.S. de Oliveira, and D. do Nascimento Marreiro, *Antioxidant role of zinc in diabetes mellitus.* World journal of diabetes, 2015. **6**(2): p. 333.

6. Myers, S.A., et al., *The zinc transporter, Slc39a7 (Zip7) is implicated in glycaemic control in skeletal muscle cells.* PloS one, 2013. **8**(11): p. e79316.

7. Tang, X.-h. and N.F. Shay, *Zinc has an insulin-like effect on glucose transport mediated by phosphoinositol-3-kinase and Akt in 3T3-L1 fibroblasts and adipocytes.* The Journal of nutrition, 2001. **131**(5): p. 1414-1420.

8. Zhang, W., et al., *MAPK/ERK signaling regulates insulin sensitivity to control glucose metabolism in Drosophila.* PLoS Genet, 2011. **7**(12): p. e1002429.

9. Wiza, C., et al., *Over-expression of PRAS40 enhances insulin sensitivity in skeletal muscle.* Archives of physiology and biochemistry, 2014. **120**(2): p. 64-72.

10. Henriksen, E.J. and B.B. Dokken, *Role of glycogen synthase kinase-3 in insulin resistance and type 2 diabetes.* Current drug targets, 2006. **7**(11): p. 1435-1441.

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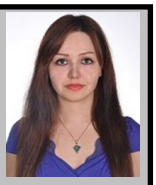
Zinc Regulates Molecules Implicated in Insulin Signaling: Implications for Insulin Resistance



Shaghayegh Norouzi and Stephen Myers

School of Health Sciences, University of Tasmania, Launceston, Australia shaghayeg.norouzi@utas.edu.au

INTRODUCTION

Insulin resistance is a common pathophysiological condition which is related to the development of chronic disorders including type 2 diabetes. Evidence shows that zinc has insulin-mimetic activity and plays an important role in glucose homeostasis. In this study, we investigated the insulin mimetic activity of zinc on insulin signalling (Figure 1).

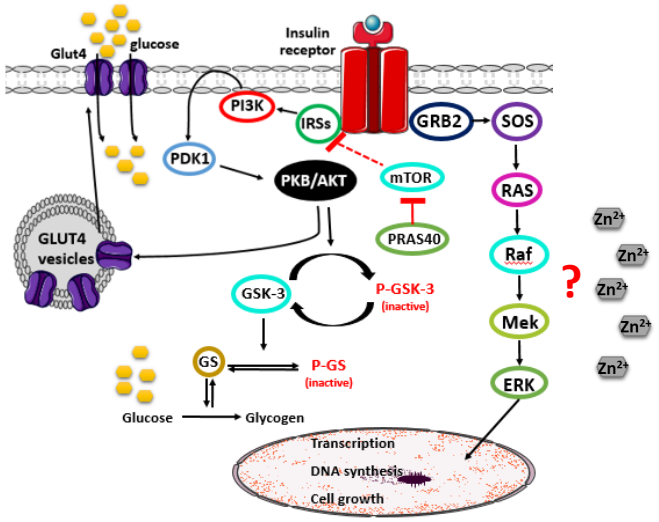
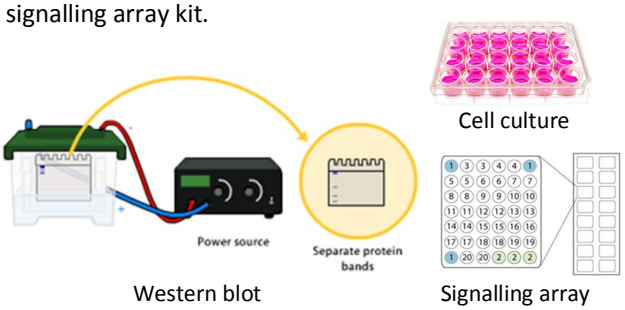


Figure 1. Schematic diagram of insulin signalling pathway pathway.

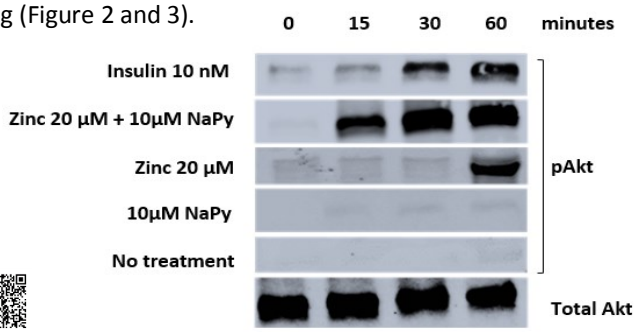
METHODS

Skeletal muscle (C2C12) cells were treated with 10 nM insulin or 20 µM zinc over 0-60 minutes and assayed for the phosphorylation of signalling molecules using western blotting and signalling array kit.



RESULTS

Zinc activates signalling molecules important in insulin signalling (Figure 2 and 3).



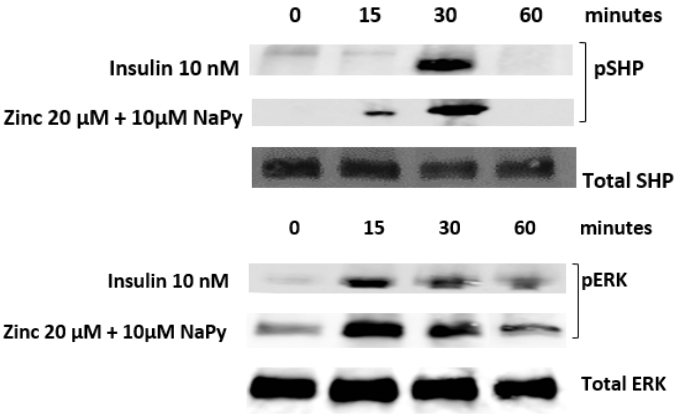


Figure 2. Western blot analysis results of Akt, SHP and ERK expression in C2C12 cells treated with zinc or insulin.

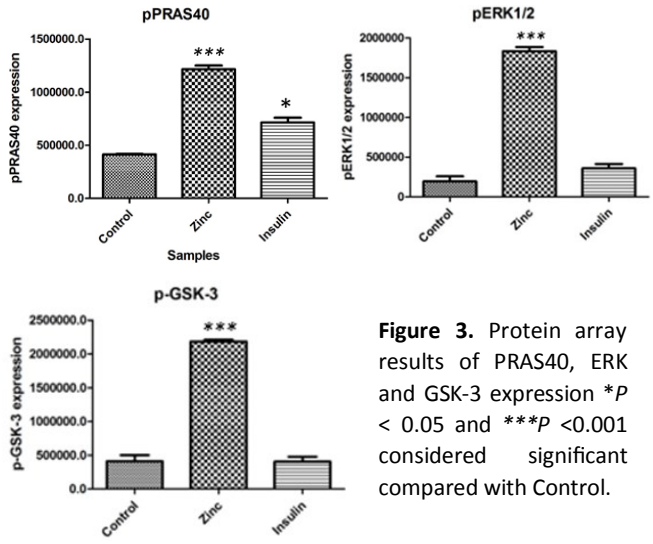


Figure 3. Protein array results of PRAS40, ERK and GSK-3 expression *P < 0.05 and ***P <0.001 considered significant compared with Control.

DISCUSSION

Our results indicate that zinc activates important molecules implicated in glucose metabolism in skeletal muscle cells. Understanding how zinc is involved in insulin signalling pathway, may present opportunities to develop novel therapies to prevent or treat insulin resistance and type 2 diabetes.

REFERENCES

1. Lee, S.R., et al., *The critical roles of zinc: beyond impact on myocardial signaling.* The Korean Journal of Physiology & Pharmacology, 2015. **19**(5): p. 389-399.

2. Fukada, T., et al., *Zinc homeostasis and signaling in health and diseases.* JBIC Journal of Biological Inorganic Chemistry, 2011. **16**(7): p. 1123-1134.

3. Myers, S.A., et al., *The zinc transporter, Slc39a7 (Zip7) is implicated in glycaemic control in skeletal muscle cells.* PloS one, 2013. **8**(11): p. e79316.

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Zinc induces the insulin signaling cascade in human skeletal muscle cell line

Shaghayegh Norouzi, John Adulcikas, Sukhwinder Singh Sohal and Stephen Myers
School of Health Sciences, University of Tasmania, Launceston, Australia

E-mail: Shaghayeg.Norouzi@utas.edu.au

INTRODUCTION

Insulin resistance (IR) is a medical disorder that is associated with the development of type-2 diabetes (T2D) (1). Previous studies have suggested that the essential trace element, zinc, is a crucial component of insulin signaling and glucose metabolism and it may delay the onset of T2D among patients with IR (2). However, the potential mechanisms by which zinc can improve insulin signaling and thus glycaemic control, are not clearly understood. Our study evaluated the insulin-like effects of zinc on the insulin signalling cascade in human skeletal muscle cells to further investigate the role of zinc in the management of IR and T2D (Fig, 1).

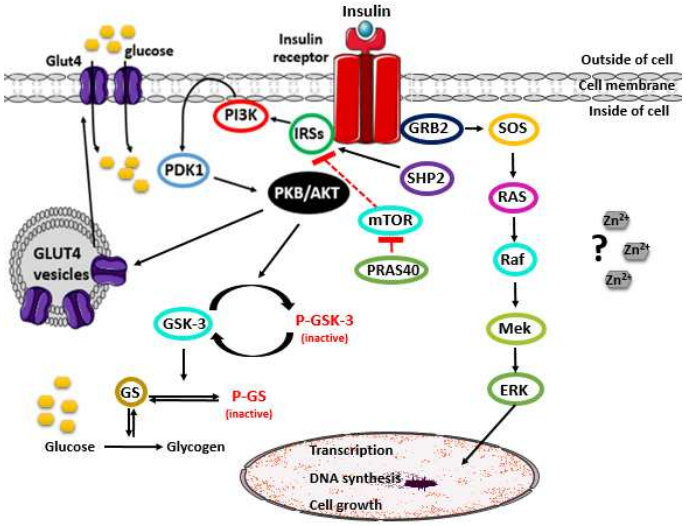


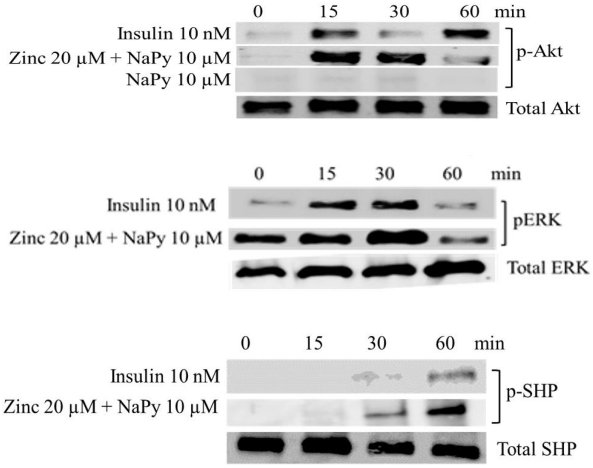
Figure 1. Schematic diagram of insulin signaling pathway.

METHODS

Human skeletal muscle cells were cultured and treated with 10 nM insulin or 20 µM zinc over 60 minutes and protein lysates were collected. Immunoblotting analysis was performed on several cell signaling proteins implicated in the insulin signaling pathway. Glucose oxidation assays were performed on skeletal muscle cells treated with insulin, zinc, or a combination of both. Data, represented as the means ± SEM, were analysed by the one-way ANOVA for multiple comparisons using the Graph Pad Prism 5 software to determine any significant differences.

RESULTS

We identified that, zinc exhibited insulin-mimetic activity on the protein expression of key markers implicated in insulin signaling including Akt, ERK1/2, SHP-2, PRAS40 and GSK3β (Fig, 2 and 3). Zinc also increased the protein expression of glucose transporter Glut-4 (Fig, 4) and increased glucose consumption in human cells (Fig, 5).



RESULTS

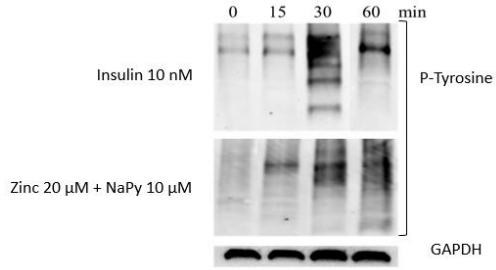


Figure 2. Analysis of Akt, ERK1/2, SHP-2 and Tyrosine in human skeletal muscle cells treated with insulin, zinc and NaPy over 60 min.

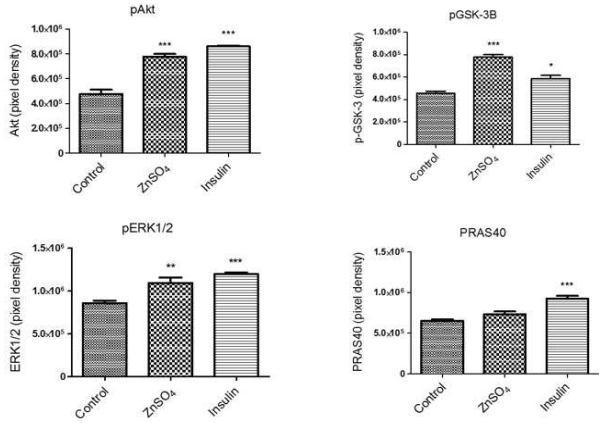


Figure 3. Densitometry results of protein signaling array in human skeletal muscle cells. *P < 0.05, **P <0.01 and ***P <0.001 considered significant compared with Control.

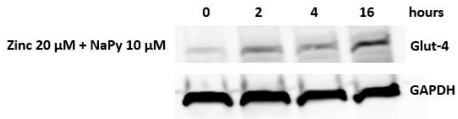


Figure 4. Analysis of Glut-4 in human skeletal muscle cells treated with zinc and NaPy over 16 hours.

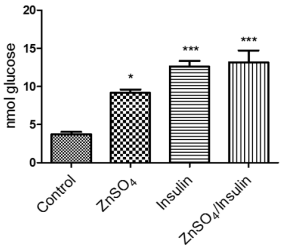


Figure 5. Glucose oxidation assay in the presence of insulin, zinc and NaPy. *P < 0.05 and ***P <0.001 considered significant compared with Control.

CONCLUSION

This study demonstrates that zinc activated the insulin signalling pathways and it was involved in glucose uptake in human skeletal muscle cells. Understanding how zinc regulates processes involved in insulin signalling may present opportunities to reduce or better manage insulin resistance and the progression of T2D.

REFERENCES

1. S Myers and S Norouzi, The Relationship between Zinc and Insulin Signaling: Implications for Type 2 Diabetes. J Clin Diabetes Pract, 2016. 1:3

2. S Norouzi, J Adulcikas, S Sohal and S Myers, Zinc transporters and insulin resistance: Therapeutic implications for type 2 diabetes and metabolic disease. JBMS 2017. Accepted